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THE PHYSIOLOGY AND CULTIVATION IN ARTIFICIAL MEDIA OF NEMATODES PARASITIC IN THE ALIMENTARY TRACT OF ANIMALS

BY A. D. HOBSON, *Department of Zoology, King's College, Newcastle-upon-Tyne*

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I. INTRODUCTION

It is intended that this review shall give an account of what is known of the physiology of the parasitic stages of nematodes parasitic in animals, with particular reference to those species which are found in the alimentary tract of their hosts. An account will also be given of attempts which have been made to keep the adult and larval phases of parasitic nematodes alive *in vitro* and of the bearing of the available physiological data upon this problem.

A number of valuable reviews dealing with various aspects of the subject have already been published. Among these may be mentioned those of Rauther (in Kükenthal, 1928–33), McCoy (1935), Peters (1936), Lapage (1937), Hoeppli, Feng & Chu (1938), Brand (1938*a*), Chitwood & Chitwood (1938), Ackert & Whitlock (n.d.) and Brand & Jahn (n.d.). To all of these the present writer is indebted for information.

The nomenclature employed in this review is, so far as possible, that of Baylis (1929).

II. THE ENVIRONMENT OF PARASITES OF THE ALIMENTARY CANAL OF VERTEBRATES

A. The digestive secretions

Because the volume of the secretions passed into the alimentary tract is so large (7,500–10,000 c.c./day for an average man, according to figures quoted by Rowntree (1922)), they must play a large part in determining the inorganic composition of its contents.

(1) *Osmotic pressure*

Data from various sources summarized by Schultz (1925) show that, with the exception of the saliva, the osmotic pressure of the digestive juices is fairly constant and very close to that of the blood. The saliva differs in that it is extremely variable and always distinctly hypotonic to the blood. Gilman & Cowgill (1931, 1933*a*, *b*) have shown that the gastric juice, bile and pancreatic juice are practically isotonic, and that an artificially produced change in the osmotic pressure of the blood is reflected in a corresponding alteration in that of the digestive juices.

(2) *Electrolyte composition*

The electolyte content of the digestive juices has been investigated by a number of workers. Among the papers which have been consulted are those of Rosemann (1907) and of Gamble & McIver (1928*a*) on the gastric secretions of the dog and the cat respectively, of Gamble & McIver (1928*b*) on the pancreatic juice of the dog, and of Reinhold & Wilson (1933) on the bile of the dog. Sobotka (1937) gives a general account of the bile. The intestinal secretions are described by de Beer, Johnston & Wilson (1935) and by Herrin (1935). The following general picture is presented by these publications.

There is only a small amount of sodium in gastric secretions (about 10 mmol.); in hepatic bile it reaches a concentration of 170 mmol. or more, while in the remaining secretions it varies between 140 and 150 mmol. Potassium (about 5–11 mmol.) and calcium (about 1·5–4 mmol.) vary comparatively little. Small amounts of magnesium have been recorded in the jejunal secretion (about 0·5 mmol.) and hepatic bile (1·8 mmol.). Chlorine is high in gastric secretion (178 mmol. for the dog) and in the jejunal secretion, but there is only about half this concentration in the pancreatic juice, the hepatic bile and the secretions of the ileum and colon. Reinhold & Wilson (1933) state that the hepatic bile of the dog contains an average of 34 m.equiv./l. of HCO_3. De Beer *et al.* (1935) measured the total carbon dioxide content of the intestinal secretions of the dog and found much variation in different individuals. In the jejunum thirteen of their observations varied from 5·2 to 30·0, in the ileum six varied from 69·8 to 97·5, and in the colon three varied from 85·8 to 93·3 m.equiv./l.

Table 1. *Composition of the contents of the small intestine of the pig*

(Analyses by Eden of samples obtained by Hobson & Stephenson, unpublished.)

Sample	Date of sample	No. of pigs	Na (mmol.)	K (mmol.)	Ca (mmol.)	Mg (mmol.)	Cl (mmol.)	P (mmol.)	Dry matter (%)	Total ash (%)
A	15. ix. 42	11	277	47·6	5·7	11·1	83	26·8	6·84	1·04
B	15. ix. 42	11	277	46·0	5·5	14·0	73	26·8	6·70	1·01
Average of samples A and B			277	46·8	5·6	12·6	78	26·8	6·77	1·03
31	5. i. 43	5	117	20·7	27·4	6·6	42	22·9	5·4	1·2
32	5. i. 43	5	119	22·2	7·2	7·0	38	23·2	5·6	1·1
33	5. i. 43	5	117	26·6	6·5	5·8	51	22·6	5·8	1·0
34	5. i. 43	5	137	35·0	2·2	4·1	88	26·4	7·8	1·1
35	5. i. 43	5	121	21·5	36·9	6·2	60	23·9	6·3	1·2
36	5. i. 43	5	134	34·0	2·0	3·3	88	26·4	7·8	1·1
Average of samples 31–36			124	26·7	13·5	5·5	61	24·2	6·5	1·1

B. The contents of the alimentary canal

(1) *Electrolyte composition*

The data summarized above show that the greatest differences in the electrolyte composition of the various digestive juices are in the sodium, chloride and bicarbonate concentrations, and to these must be added the differences in hydrogen-ion concentration. The potassium, calcium and magnesium contents do not vary greatly. Since the discharge of the digestive secretions is not a continuous, but a periodic process, and since food and feeding habits are also variable, the conditions in each part of the alimentary canal must vary considerably from time to time. Table 1 shows the results of analyses carried

out by Dr A. Eden, of the Ministry of Agriculture and Fisheries Veterinary Laboratory, Weybridge, on samples of material taken from the small intestine of pigs by Hobson & Stephenson (unpublished). The samples were obtained on two different dates from freshly slaughtered animals. Each consisted of a mixture of approximately equal volumes of the intestinal contents of a number of pigs. They give some idea of the amount and type of variation encountered. The extraordinarily high values for sodium and potassium in the first pair of samples and the great variability in the calcium concentrations of the second group are particularly noteworthy. As might be expected, the chloride values also display a considerable degree of variation.

from 7·0 to 7·6 during fasting and from 6·2 to 6·8 5–6 hr. after feeding. The ileum was generally alkaline in the fasting animal (*p*H 7·0–8·0) and slightly more acid (as low as *p*H 6·5) after a meal. The reaction of the colon was usually about *p*H 7·4 while the animal was fasting; after a meal rich in protein or after a mixed diet there was only a very slight decrease in the *p*H after 4–6 hr., but carbohydrate food induced a fall to *p*H 6·0 or even, exceptionally, to *p*H 4·4.

Variations as wide as these cannot be ignored when we are considering the environmental conditions of intestinal parasites. Some species, however, may not be subjected to such great fluctuations. Kofoid, McNeil & Cailleau (1932) demonstrated con-

Table 2. *Depression of the freezing-point of gut contents*

Material	Δ (° C.) Maximum	Minimum	Average	Author
Horse (intestine)	—	—	0·69	Vialli (1923) (quoted by Schopfer, 1932)
Horse (intestine)	0·850	0·674	0·755	Schopfer (1924)
Horse (intestine)	—	—	0·69	Duval & Courtois (1928)
Pig (intestine)	1·30	0·73	1·02	Schopfer (1932)
Pig (intestine)	0·940	0·733	0·869	Hobson & Stephenson (unpublished)
Sheep (intestine)	—	—	0·815	Schopfer (1932)
Sheep (abomasum)	0·610	0·555	0·579	Davey (1936)
Scyliorhinus caniculus (intestine)	2·65	2·30	—	Schopfer (1927*b*)
Scyliorhinus caniculus (intestine)	—	—	*c.* 2·40	Schopfer (1924)

Note. In the table given above the averages of Schopfer's determinations are those of his published determinations. In Table 2 of his paper published in 1927(*b*) he gives the following as representative figures for the intestinal contents: horse 0·75°, pig 0·9–1°, sheep 0·82°. The average figure for the abomasum of the sheep is calculated from Davey's Table 1 (1936).

(2) *Hydrogen-ion concentration*

The hydrogen-ion concentrations in the alimentary tract have received a great deal of attention, and their general character is so well known that it is not necessary to discuss this subject in detail. It is important to note, however, the considerable degree of variation which may occur in any particular region. Mann & Bollman (1930), for example, made extensive observations on the dog and found that, in the stomach, the *p*H might vary between 1·5 and 6·9 even in the fasting animal. Immediately after a meal the contents of the stomach usually approached neutrality, but rapidly became more acid, reaching about *p*H 1·5 2–4 hr. later 'and remained in this neighbourhood for the rest of the day, except for a few brief periods of neutralization, in the course of which it sometimes rose as high as *p*H 4·0'. In the duodenum, while the dog was fasting, the *p*H was about 7·6, but after a meal there was a fluctuating decrease to about *p*H 5·5 and, after 2 hr., even as low as *p*H 4·6. After this there was a gradual rise to about *p*H 6·8, and later still, 6–12 hr. after the meal, the fasting value was attained. The jejunum showed a smaller degree of variation. Usually the *p*H varied considerable differences between the hydrogen-ion concentration of the contents of the various parts of the alimentary canal of the rat and that of scrapings from its wall. On the whole their records of the *p*H of the contents seem to vary between wider limits than those of the scrapings of the wall. Robinson (1935) passed isotonic $CaCl_2$ solutions through jejunal and ileal loops of dogs and found that each part of the intestine tended 'to establish in its contents its own characteristic reaction', irrespective of that of the original solution used. He suggested that there is a zone at the surface of the mucosa where the reaction is stabilized, and that this value is not necessarily given by measurement of the *p*H of the whole intestinal contents at that point. If this is true, parasites living in close contact with the mucosa are exposed to more constant conditions than those which are free in the gut contents.

(3) *Osmotic pressure*

Measurements of the osmotic pressure of the contents of the alimentary canal are not numerous, and the majority have been made on material taken from the intestine. Table 2 summarizes the data and

shows that, in the intestine, although there is a considerable range of variation, the values of Δ are appreciably greater than those of mammalian blood (see, for example, Collip, 1920). This also holds good for *Scyliorhinus caniculus*, in which Schopfer (1927*b*) found the depression of the freezing-point of the blood to be 2·10° C., in contrast to values varying between 2·30 and 2·65° C. in the intestinal contents. The gut contents are not, however, invariably hypertonic to the blood. Davey (1936), working with lambs 4 months' old, found that the freezing-points of the blood and of the contents of the rumen, reticulum and abomasum were closely similar.

Table 3. *Oxygen content in fluid intestinal masses*

Animal	Part of intestine	Oxygen in volumes %. Mean and (extremes)	No. of determinations	Investigator
Horse	Small intestine	0·024 (0·016–0·031)	2	Toryu (1934)
Dog	Small intestine	0·028	1	Brand & Weise (1932)
Cattle	Small intestine	0·013 (0·00–0·025)	2	Brand & Weise (1932)
Sheep	Small intestine	0·012 (0·00–0·025)	4	Brand & Weise (1932)
Pig	Small intestine	0·083 (0·00–0·358)	6	Brand & Weise (1932)
Cattle	Large intestine	0·010 (0·00–0·023)	3	Brand & Weise (1932)
Pig	Large intestine	0·00	1	Brand & Weise (1932)

Table 4. *Oxygen content of intestinal gases*

Animal	Part of intestine	Oxygen in volumes %. Mean and (extremes)	No. of determinations	Investigator
Horse	Small intestine	0·67 (0·57–0·76)	3	Tappeiner (1883)
Cattle	Small intestine	0·00	1	Tappeiner (1883)
Pig	Small intestine	5·5 (1·2–14·2)	9	Long & Fenger (1917)
Pig	Small intestine	4·2 (0·4–8·2)	6	Brand & Weise (1932)
Dog	Small intestine	0·2 (0·0–0·7)	8	Planer (1860)
Horse	Large intestine	0·07 (0·00–0·14)	4	Tappeiner (1883)
Cattle	Large intestine	0·00	1	Tappeiner (1883)
Goat	Large intestine	0·03 (0·00–0·07)	3	Tappeiner (1883)
Sheep	Large intestine	0·00	1	Tappeiner (1883)
Rabbit	Large intestine	0·62	1	Tappeiner (1883)
Dog	Large intestine	0·00	6	Planer (1860)

(4) *Gases*

The available data on the gases present in the intestine have been summarized and discussed by Brand (1938*a*) and by Brand & Jahn (n.d.). The dissolved oxygen was measured by Brand & Weise (1932) in the dog, cattle, sheep and pig and by Toryu (1934) in the horse. Their results show that although in some cases no oxygen is present, there is usually a small amount. Table 3 is taken from Brand (1938*a*, table 1).

Oxygen may also be a constituent of the undissolved gas found in the intestine. The composition of these gases has received the attention of various workers, and the data summarized in Table 4 have been extracted from the review by Brand & Jahn (n.d., table 4).

It seems, therefore, that parasites living in the small intestine have access to a small amount of oxygen, estimated by Brand & Jahn as about 5% of saturation, except in the pig, in which species it is somewhat higher. We do not know with certainty whether this small amount can be utilized

by intestinal nematodes, but some evidence derived from the behaviour of worms kept under experimental conditions at low oxygen tensions indicates that it may be (see, for example, Harnisch (1933) and Krüger (1936)).

Other gases which may be present in extremely variable quantities are nitrogen, hydrogen, methane and carbon dioxide. It is improbable that the first three of these have any physiological action on the worms, but Brand & Jahn (n.d.) suggest that, since the intestinal contents are usually saturated with carbon dioxide, this substance may play an important part in the regulation of the intracellular hydrogen-ion concentration of the parasites.

(5) *The bile*

The bile contains, in addition to inorganic salts, considerable but variable amounts of mucin and pigment and of lipids, including cholesterol. A summary of the available data is given by Sobotka (1937). There is also much variation in the nature and proportion of the bile salts present (see Sobotka, 1937, table 8). In some animals, such as man and the ox, both glycocholate and taurocholate are present; in many species, such as the sheep, the goat and the dog, only taurocholate is present; in a few, such as the pig, only glycocholate occurs.

According to Dopter & Deschiens (1938), Chabrol (1937) estimated the total bile salts concentration in the duodenal contents of man at about 15 parts per 1000. On the basis of the figures of von Zeynek (no reference given), that human gall bladder bile contains 27·4 parts per 1000 of taurocholate and 69·5 parts per 1000 of glycocholate, Dopter & Deschiens calculate that the concentrations of these two salts in the duodenal contents should be 4·5 and 10·5 parts per 1000 respectively.

III. THE CHEMICAL COMPOSITION OF NEMATODES

(1) *Dry weight*

Several workers have measured the proportion of dry substances in the whole body of the worms. Their results are summarized in Table 5.

The values for total dry weight given by Flury are markedly less than those of other workers. It is possible that this is due to a difference of method since he dried his material over sulphuric acid at room temperature. It is noteworthy that even if we include Flury's determinations (see Table 6) no individual tissue shows a dry weight as low as 15 %, with the exception of the body fluid. The figures available for the separate tissues are given in Table 6.

(2) *General analyses*

The majority of analyses of whole worms, or of particular organs, have been made with the object of investigating the problems of carbohydrate and fat metabolism. These will be discussed in a later part of this review. Only the general and certain special features will be considered here.

The most complete analyses of the entire body are those of Flury (1912). His results are given in Table 7.

With regard to the figures quoted in Table 7 it should be remembered that they are based upon an unusually small solid content for the whole body of the animal. Judging by the values obtained by other workers shown in Tables 5 and 6 the dry weight of *Ascaris* is about 20 % or more of the total weight.

Qualitative tests by Flury (1912) showed the presence of glycocoll, valine, leucine, asparagine, arginine, histidine and lysine. Tyrosine was found

Table 5

Species	Sex (if specified)	Dry weight as percentage of total body weight: Maximum	Minimum	Average	Author
Ascaris lumbricoides	—	21·5	19·9	—	Weinland (1901*b*)
A. lumbricoides	—	21	14	15	Flury (1912)
A. lumbricoides	—	24·88	22·73	23·75	Schulte (1917)
Parascaris equorum (small specimens)	—	—	—	20·626	Schimmelpfennig (1903)
P. equorum (larger specimens)	—	—	—	21·952	Schimmelpfennig (1903)
P. equorum	—	18·30	12·9	14·77	Flury (1912)
P. equorum	♀	20·720	19·041	19·484*	Toryu (1933)
P. equorum	♂	24·114	20·000	22·755*	Toryu (1933)
Larval *Eustrongylides ignotus* from *Fundulus heteroclitus*	—	28	22	25	Brand (1938*b*)

* These averages calculated from published figures.

after acid hydrolysis. Uric acid and creatinine were apparently absent.

Yoshimura (1930) made quantitative analyses of the material obtained after hydrolysis of the entire bodies of *A. lumbricoides* from man. His results are given in Table 8.

Flury (1912) also analysed the ash of *A. lumbricoides* and his results are quoted in Table 9.

Table 6

Species	Organ	Dry weight as percentage of wet weight of organ: Maximum	Minimum	Average	Author
Ascaris lumbricoides	Body wall	25·0	23·5	—	Flury (1912)
	Alimentary canal	—	—	27·50	
	Reproductive organs	33·3	25·0	—	
	Body fluid	6·70	4·0	—	
A. lumbricoides	Cuticle	27·10	27·01	27·05	Hobson, Hobson, Bernacca & Stephenson (unpublished)
	Contractile part of muscle layer and hypodermis	26·02	24·20	24·84	
	Non-contractile part of muscle layer	27·58	24·15	25·26	
	Alimentary canal	22·25	17·67	20·01	
	Ovary + oviduct	31·30	26·77	29·78	
	Uterus	19·63	17·85	18·48	
	Body fluid	6·71	5·80	6·48	
Parascaris equorum	Body fluid	—	—	8·27	Marcet (1865)
P. equorum	Body wall	—	—	25·0	Flury (1912)
	Alimentary canal	—	—	24·90	
	Reproductive organs	27·4	24·0	—	
	Body fluid	—	—	8·27	

Table 7. *Composition of* Ascaris lumbricoides (*Flury*, 1912)

	Percentage dry substance	Percentage fresh material on basis of 85 % water content
Protein	54·25	8·138
Purine bases	0·46	0·069
Fat and lipoid	10·88	1·632
Lecithin (included in last)	0·72	0·108
Carbohydrate and 'Extraktstoffe'	29·78	4·467
Glycogen (included in last)	24·40	3·650
Mineral	5·09	0·763
Water and volatile substance	—	85·000

Table 8 (from Yoshimura, 1930)

	Hydrolysis with H_2SO_4 percentage dry substance	Hydrolysis with HCl percentage dry substance
Leucine	15·54	3·70
Alanine	—	1·45
Valine	—	0·79
Proline	—	3·41
Isoleucine	—	1·45
Serine	—	0·72
Glutaminic acid	—	3·93
Aspartic acid	—	0·36
Glycocoll	—	0·29
Phenylalanine	—	0·02
Histidine	0·45*	—
Arginine	1·28*	—
Lysine	2·58*	—
Tyrosine	2·09	—
Purine base N	0·03*	—

* Average figures.

These analyses of the whole body do not, however, tell us very much, beyond giving a general picture of an animal with a fairly high solid content, an unusually large amount of carbohydrate, especially glycogen, and a considerable reserve of fat. For further information we must look to the examination of individual organs and groups of organs. About these, unfortunately, comparatively little is known, except with regard to their fat and carbohydrate contents. These will be discussed later.

(3) *The cuticle*

Although a number of workers have applied chemical methods to the investigation of the cuticle, the major part of our information concerning it is derived from histological studies. The cuticle is an extremely complicated structure. Goldschmidt (1904) distinguished histologically eight component elements in the cuticle of *A. lumbricoides*, while

Chitwood & Chitwood (1937) described nine. There is a good deal of variation in the structure of the cuticle in different species of nematodes, but some of the characteristic layers can be recognized throughout the group. For a comparative account reference should be made to the article by Chitwood & Chitwood (1937).

It seems probable that so complex a structure as the nematode cuticle should also be complex in its chemical composition, and this is borne out by recent investigations.

Table 9. *Inorganic constituents of* Ascaris lumbricoides *on the basis of an average ash content of* 5·0 % *of the dry substance and* 0·75 % *of the living animal* (*Flury*, 1912)

	In dry substance (%)	In fresh material (%)
Na	1·104	0·1656
K	0·607	0·0910
Ca	0·404	0·0606
Mg	0·058	0·0086
Al	0·131	0·0197
Fe	0·019	0·0028
Cl	1·272	0·1909
PO_4	1·315	0·1973
SO_4	0·114	0·0171
SiO_2	0·029	0·1044

The cuticle of nematodes has often been described in the past as being composed of chitin, but the fact that it is incorrect to apply this term to it was established long ago by the work of Lassaigne (1843) and Sukatschoff (1899), both of whom showed that the cuticle is soluble in potassium hydroxide. According to Flury (1912), Reichardt (1902) found that the outermost layer of the cuticle of *A. lumbricoides* and *Parascaris equorum* consists of protein materials rich in sulphur which do not yield sugar or any other reducing substances when treated with dilute sulphuric acid. Chitwood (1936) states that Reichardt found the cuticle to be insoluble in hot or cold water, but that all except the outermost (cortical) layer dissolved when heated with water in a closed tube to 140° C. The cortical layer was more resistant than the internal layers to concentrated hydrochloric acid and differed from them in resisting digestion by gastric and by pancreatic juice. Material from the inner layers gave positive Millon, xanthoproteic and biuret tests. The cortical layer gave a negative biuret, but the other two tests were positive.

Flury (1912) was the first to call attention to the high sulphur content of the cuticle. He obtained the material with which he worked by incubating the cuticle with pepsin under toluol for several days. Presumably, as Chitwood (1936) remarks, this material consisted of the cortical layer and the fibrils connected with it and not of the whole cuticle. This material was insoluble in water, alcohol and ether, but soluble, except for some fine threads ('einige faserige Flöckchen'), in warm, dilute sulphuric acid and in hot potassium hydroxide. Concentrated sulphuric acid caused a slow disintegration into a fine felt, which underwent no further change.

The original digest, after heating with 10 % HCl and neutralization, gave positive xanthoproteic, biuret and Millon tests. Other tests indicated the presence of tryptophane, histidine, cystine or unoxidized sulphur and carbohydrate. Further purification removed the carbohydrate, but the tryptophane test became still more marked. The yield of the purified substance dried *in vacuo* to constant weight was 1–1·5 g./1000 g. worms, not per 100 g. worms as stated by Chitwood & Chitwood (1937). The results of the elementary analysis are given in Table 10. Flury concluded from the high sulphur content and other features of its behaviour that the cuticle of the ascarids is a keratin.

Table 10. *Elementary analysis of dried and purified cuticular substance according to Flury* (1912). *Note that presumably it includes only cortical layer and fibrils*

	Sample I (%)	Sample II (%)
C	50·010	51·13
H	5·560	6·62
N	15·730	16·73
S	4·607	4·12
O	23·803	21·10
Ash	0·290	0·30
	100·000	100·00

Magath (1919) worked with the whole cuticle of *Camallanus americanus* washed and dried to constant weight. It was insoluble in diluted mineral acids, but dissolved in concentrated acids, in caustic alkalis even as dilute as 1 %, and in ammonia. It swelled in acetic acid and partly dissolved in boiling water. The xanthoproteic, Hopkins-Cole and unoxidized sulphur tests were positive, but the biuret test was feeble or negative. There was no reduction with Fehling's solution. Urea, uric acid and creatine were absent. Cystine was present in the filtrate obtained after boiling the cuticle for several hours in water. In the whole cuticle there was 16·90–17·04 % nitrogen and 1·16–1·25 % sulphur. Magath concluded that the protein is an albuminoid.

Mueller (1928) scraped away the tissues from the cuticle of *Ascaris lumbricoides*. The latter was then washed in distilled water and heated in an autoclave for 20 hr. at 12 lb. pressure. This separated the soluble inner layers from the insoluble outer part. The solution, when it was evaporated, yielded a glue-like substance which gave no protein reactions. This result indicated that these substances had been hydrolysed. The inner soluble portion formed about

five-sixths of the whole cuticle. Nitrogen estimates gave average values of 15·91 % for the inner and 16·09 % for the outer layer. Owing to lack of material, sulphur determinations were only made on the material of the inner layers, which contained 0·820 % of sulphur. Mueller points out that, if the sulphur estimations of Flury and of Magath already quoted are correct, the value for the outer layer must be much greater than for the inner.

Mueller criticized severely Magath's conclusion that the protein of the cuticle is an albuminoid of the collagen, gelatin, elastin group. He always obtained a positive Millon reaction indicating that tyrosin is present, while Magath denied this. He was also unable to confirm the swelling effect of immersion in acetic acid observed by Magath. Mueller's conclusion is that two proteins are present, and that the question of their relationships should be left open until further evidence is obtained.

A more extensive study of the proteins of the cuticle of *A. lumbricoides* has been made by Chitwood (1936). He found that pepsin digests all except the cortical and fibril layers. Digestion experiments with various types of 'artificial pancreatic juice' indicated the presence in the cuticle of at least three distinct substances. The following colour reactions carried out on the whole cuticle were all positive: xanthoproteic, mercuric nitrate (modified Millon), aldehyde, biuret, arginine (Sakeguchi), lead acetate in 5 % NaOH (for unoxidized sulphur), Molisch and barium chloride (for oxidized sulphur).

Following these experiments attempts were made by various methods of treatment to segregate the types of substance present and to discover to which part of the cuticle each belongs. Five such segregates were prepared (for details of the preparation of these reference should be made to the original paper) and the following proteins were distinguished: albumins and, probably, a glucoprotein neither of which 'correspond to known morphological entities'; a fibroid (matricin) corresponding to the matrix layer; a collagen (ascarocollagen) corresponding to the fibre, internal cortical and fibre layers; a keratin corresponding to the external cortical layer.

In the cuticle of a very different type of nematode, *Ditylenchus dipsaci*, Chitwood (1938*b*) found a similar protein composition and brought forward some evidence of the existence of another type of substance on the outside of the cuticle. He found that exposure to NaOCl dissolved the whole of the cuticle with the exception of 'an exceedingly delicate membrane which dissolves in acetone, alcohol and 10 % NaOH (24 hr.) but does not stain in osmic acid, Scharlach R or Nile blue sulphate. It is absent after specimens have been treated to 65° C.' He suggests that this outer 'thermolabile layer' is possibly composed of a wax or a sterol. A similar substance forms a layer in the cuticle of *Rhabditis strongyloides* and *Oncholaimium oxyuris* and is also present in the eggs of *Ditylenchus dipsaci* and of *Heterodera marioni*. He suspects that such a cuticular layer occurs generally among nematodes.

It will be seen, from the evidence summarized above, that the cuticle of nematodes is an exceedingly complex structure, both morphologically and chemically. The existence of at least five types of protein seems to be established by the work of Chitwood. One of these, forming the outer cortical layer, is probably a keratin, and this conclusion is supported by the earlier investigations of Flury and by what is known of its staining properties. The thermolabile wax or sterol layer of Chitwood requires further investigation and has not so far been demonstrated in a nematode parasitic in animals. If it is present in these species it is, as Chitwood (1938*b*) points out, of great importance in connexion with the penetration of anthelminthic substances, which should be lipoid soluble if they are to pass through the body wall.

(4) *The alimentary canal*

The intestine of a nematode such as *Ascaris lumbricoides* is formed of a single layer of columnar epithelial cells. Their structure is extremely uniform and has been described in detail by Hirsch & Bretschneider (1937*a*). The inner surface of the intestine is covered by a peculiar and conspicuous layer variously called the bacillary layer, *Stäbchensaum* or *bordure en brosse*, whose nature has been the subject of much speculation and is still uncertain. Among various opinions which have been expressed, perhaps the most likely is that supported by Hetherington (1923) that the bacillary layer is composed of fused cilia whose basal granules are represented by the deeply staining subbacillary layer. The function of the bacillary layer is not yet understood. Chitwood & Chitwood (1938) expressed the opinion that the function of this layer 'is a problem for general zoology rather than nematology'. They point out that the bacillary layer 'occurs in the intestine of various groups of worms as well as in arthropods and vertebrates'. Whether the structures to which they refer are strictly comparable with the bacillary layer of nematodes has yet to be proved.

On the outer surface of the intestine there is a thin but distinct 'cuticular' layer, the basal lamella. It is well developed in *Ascaris* but is not present in all nematodes. Its origin, function and chemical nature are unknown, but it stains with collagen stains (Chitwood & Chitwood, 1938; Hobson & Hobson, unpublished).

The inner lining of the stomodaeum and oesophagus is of a different nature from that of the intestine. Imminck (1924), quoted by Chitwood (1938*b*), made a number of chemical tests on

Strongylus equinus and concluded that this lining is composed of a chitinoid containing protein. Chitwood (1938*b*) examined material from the same species and eliminated the possibility of its being chitin, keratin or fibroid. He considered it to be collagen containing mucoids.

The intestinal cells of *Ascaris* contain glycogen and fat (Kemnitz, 1912; Fauré-Fremiet, 1913*a*; Hirsch & Bretschneider, 1937*a*). These substances and their significance will be discussed in later sections of this review. On the basis of spectroscopic examination, Flury (1912) reported the presence of haemoglobin in the intestinal cells of *Parascaris equorum*, and Fauré-Fremiet (1913*d*) considered that this had been derived from the host. Lièvre (1934), however, was unable to confirm Flury's observation. Flury also observed a green pigment, but he failed to identify this with bile pigments by the Gmelin and Hammarsten tests. Fauré-Fremiet, on the other hand, obtained a very feeble Gmelin reaction after dissolving the pigment in amyl alcohol.

It has been known for some time that reddish or yellowish brown birefringent granules occur in the intestinal cells of *Ascaris* and a number of other parasitic nematodes. These were regarded by Askanazy (1896), Looss (1905) and Fauré-Fremiet (1913*a*) as products derived from absorbed haemoglobin. This is uncertain, because their presence does not seem to be necessarily connected with a blood-feeding habit (see Chitwood & Chitwood, 1938). Other workers, such as Kemnitz (1912), considered them to be zymogen granules. Quack (1913) concluded that they were composed of gypsum, and this view has been supported by Chitwood & Chitwood (1938), who also demonstrated the presence of iron in these sphaerocrystals.

Rogers (1940*b*) analysed the intestine of *Strongylus edentatus* and demonstrated the presence of iron, zinc and sulphur. The amounts varied from a trace to 0·20 % of the total weight of the worm in the case of iron, a trace to 0·77 % for zinc and 0·18–0·40 % for sulphur. A small amount of copper with a trace of silver also occurred in one case.

Hill & Smyth (1944) and Smyth, Bingley & Hill (1945), using the silver nitrate-acetic acid technique, showed the presence of considerable amounts of vitamin C in the intestinal cells of *Toxocara canis*. Only traces were found in other parts of the body.

(5) *The hypodermis, muscular layer and nervous system*

The only analyses which have been made of these parts of the body are of their glycogen and fat contents. These will be discussed later.

(6) *Reproductive system*

In 1883 van Beneden described certain bodies occurring in the oocytes under the name of *boules hyalines*. Fauré-Fremiet (1913*d*) found them to consist of calcium phosphate with protein and that they are surrounded by a layer of lipoid. The calcium phosphate forms about 0·4–0·6 % of the dry weight of the ovaries.

Fauré-Fremiet (1913*c*) found that the cytoplasm of the primary spermatocytes is full of refractive granules (*grains brillants*). He isolated these from the cells and identified them as an albuminoid protein containing 17·5 % nitrogen but no sulphur or phosphorus. Later these granules fuse together to form the refringent body of the adult spermatozoon. This substance he named *ascaridine*. Later work on this protein (Fauré-Fremiet & Filhol, 1937) showed that, while it is insoluble in cold water, it dissolves suddenly at 50–51° C. The 'temperature of dispersion' is independent of the concentration of the protein and can be raised or lowered by the presence of salts, whose action depends upon their position in the lyotropic series of anions. According to the X-ray investigations by Champetier & Fauré-Fremiet (1937), ascaridine is semi-crystalline when it is precipitated from solution, but becomes amorphous if the globules are consolidated by centrifugation or pressure and desiccation.

(7) *The body fluid*

Observations on the body fluid have been made chiefly on the body fluid of the large species, *Ascaris lumbricoides* and *Parascaris equorum*. The earliest appear to have been those of Marcet (1865) on fluid obtained from the latter species. Without giving figures, except for the dry weight, Marcet commented on the small amount of chloride, the presence of considerable amounts of potash and phosphoric acid and the absence of sulphate.

Flury (1912) made a fuller analysis of the fluid from *P. equorum* and obtained the results shown in Table 11. Fauré-Fremiet (1913*a*) found albumoses and peptones in the body fluid.

Table 11. *Analysis of the body fluid of* Parascaris equorum (*Flury*, 1912)

	In fluid (%)	In dry substance (%)
Water	94·853	—
Dry substance	5·147	—
Minerals	0·923	18·46
NaCl	0·364	6·28
Purine bases	0·033	0·66
Alcohol-soluble substances	0·366	7·32
Albumin	3·514	70·28
Globulin	0·574	11·48
Proteins	4·088	81·76

Schopfer (1926*a*) made a few determinations of the chloride content of the body fluid of *P. equorum* and obtained an average value of 1·20 g. NaCl/l.

Duval & Courtois (1928) repeated these observations on material from the same species and obtained the considerably higher average value of 3·41 g. NaCl/l. Like Marcet (1865), both Schopfer and Duval & Courtois commented on the unusually small amount of chloride in the body fluid. Duval & Courtois also found high concentrations of bicarbonate (58 c.c. total CO_2/100 c.c. body fluid) and amino-nitrogen (0·60 g./l. body fluid). They found that these, together with the chloride, accounted for 68 % of the total molecular concentration of the fluid, which was estimated from a freezing-point depression of 0·57° C.

Values for glucose, protein and non-protein nitrogen, sodium and chloride contents of the body fluid of fresh *Ascaris lumbricoides* have been given by Rogers (1945), and fuller analyses of the inorganic constituents have been made by Eden in collaboration with Hobson & Stephenson (unpublished). Average figures, together with osmotic pressure and electrical conductivity determinations carried out by Hobson & Stephenson, are given in Table 12. Recalculated data from other sources are also included for comparison.

Rogers (1945) also examined the body fluid of *A. lumbricoides* kept *in vitro* for 20–24 hr.; he gives values for the substances mentioned above and for lead, zinc, potassium, magnesium, iron, haematin, ascorbic acid, amino-glucose and uronic acid. In view of the large amount of ascorbic acid found by Smyth *et al.* (1945) in the intestinal cells of *Toxocara canis*, it is interesting to note, as Rogers points out, that its concentration in the body fluid of *Ascaris lumbricoides* is about the same as in normal blood.

These analyses show several important features. The body fluid contains an unusually large concentration of potassium and magnesium, and the chloride is, in accordance with the results of earlier workers, very low, although Schopfer's figure seems to be distinctly too small. There is also a large discrepancy between the total cations and anions which may be partly accounted for by the observations of Duval & Courtois (1928) previously referred to. It should be realized that the analyses of the body fluid of *A. lumbricoides* were analyses of the total amounts of the various elements and were not confined to the inorganic and ionized constituents.

Marcet (1865) found the body fluid of *Parascaris equorum* to be slightly acid. Hobson, Stephenson & Beadle (unpublished) made electrometric determinations of the hydrogen-ion concentration of the body fluid of *Ascaris lumbricoides* and obtained initial *p*H values varying between 6·63 and 7·24. The explanation of this variation probably lies in the presence of volatile acid in the fluid. Exposure of the body fluid from one nematode to the air for 15 min. led to a progressive rise from *p*H 6·92 to 7·32.

IV. THE RELATION OF THE PARASITIC NEMATODE TO THE CHEMICAL AND PHYSICAL CONDITIONS OF ITS ENVIRONMENT

(1) *The permeability of the cuticle, body wall and alimentary canal*

Since the cuticle of nematodes is complex and, in some species, relatively thick, it is not surprising that it has been widely supposed to be the main factor responsible for the well-known powers of resistance of these animals to unfavourable environmental factors. There is, however, good evidence that the cuticle is permeable to water as well as to certain salts, non-electrolytes and dyes. Permeability to water and salts will be discussed in the next section of this review.

Little work seems to have been done on the penetration of dyes. The subject is discussed briefly by Chitwood & Chitwood (1937) who quote the work of Chitwood (1930), which showed that *intra-vitam* stains tend to penetrate rhabditid nematodes 'much more readily at the normal body openings and at thin places in the cuticle'. These authors also mention unpublished work on *Agamermis decaudata* by Christie who found a much greater permeability to *intra-vitam* stains in the parasitic than in the non-parasitic stages of the life history of this species. Chitwood & Chitwood (1937) find a greater permeability to stains in the first-stage larvae of rhabditoids and strongyloids than in later stages. Filipjev (1934) states that an *intra-vitam* stain such as methylene blue can penetrate the cuticle of a free-living marine nematode; in a fresh-water species the penetration is much slower and principally through the thinner cuticle of the papillae and by way of the mouth, anus and vagina. 'In the intestinal parasites or in the saprozoic forms, the stain penetrates only through these openings, being chiefly ingested through the mouth, the cuticle being quite impermeable to the stain.'

Preliminary experiments (unpublished) have been carried out by Hobson & Stephenson on the penetration of a number of dyes into normal and ligatured *Ascaris lumbricoides*. Only neutral red, safranin and Nile blue sulphate were found to pass through the body wall. Penetration of methylene blue, light green and brilliant cresyl blue occurred only in unligatured worms, and seemed to take place through the wall of the alimentary canal which also appeared to afford a readier passage to dyes of the first group.

Mueller (1928) introduced urea, potassium iodide and glucose dissolved in saline into cylinders composed of eviscerated sections of the body of *A. lumbricoides* immersed in 1 % saline and studied the passage of these substances through the body wall. Urea and potassium iodide quickly appeared in the outer liquid, but glucose did not pass through unless

its concentration inside the cylinder was raised to 5%, when a small amount could be detected after 10 hr. Both neutral red and methylene blue could pass outwards through the body wall.

Experiments have been carried out by Hobson & Stephenson (unpublished) on the permeability of isolated cylinders of the cuticle of *A. lumbricoides* from which all the body-wall tissue had been removed by scraping. These show that the cuticle is permeable to glucose and to a wide range of other non-electrolytes. The relative permeability to twenty-three substances was studied. In general it was found that the rate of penetration decreased with increasing molecular weight and that there was a fairly close correspondence with the results obtained by Collander (1926) with collodion membranes.

Trim (1944) found that hexyl resorcinol can penetrate readily through the body wall of *A. lumbricoides* even after all external apertures have been eliminated by ligaturing. Even in specimens which had approximately the same surface area he found large variations in the rate of uptake of the drug. The rate of penetration was roughly proportional to the concentration in the medium and was not altered over the *p*H range 4·1–7·8 or by the presence or absence of oxygen. Between 20 and 40° C. Q_{10} was about 2, but below 20° C. it rose sharply. Penetration was affected by the presence of certain substances in the medium. It was decreased by bile salts, soaps and gastric mucin and increased by a certain range of concentrations of sodium oleate below 0·25%.

(2) *The relation between the electrolyte composition and osmotic pressure of the body fluid and of the environment*

Average figures for osmotic pressure, conductivity and the concentration of sodium, potassium, calcium, magnesium, chlorine and phosphorus in the body fluid of *Ascaris* have been given in Table 12. This fluid is, however, by no means as constant in its composition as the blood of a vertebrate. There is, indeed, a considerable degree of variation in the concentration of certain of the ions present. In the seven samples of body fluid extracted from fresh *Ascaris* and analysed by Eden, the concentration of sodium varied from 118 to 138 mmol. and that of potassium from 13·8 to 37·1 mmol. The chlorine was also variable (46·3–57·0 mmol.), but the concentrations of the other ions did not fluctuate greatly. Nor is the osmotic pressure as constant, as earlier workers have also found. Schopfer (1925) was the first to point out the significant fact that, in *Parascaris equorum*, the individual variation of this factor is less in specimens obtained from the same horse than in specimens from different hosts. This observation suggests that, at any rate so far as osmotic pressure is concerned, there is a close relation between the body fluid of the worm and the external environment which is, as is shown in Table 2, subject to considerable variation. Moreover, it is probable

Table 12. *Composition of the body fluid of fresh animals*

Material	Sp.gr.	Total solids (%)	Total ash (%)	Na (mmol.)	K (mmol.)	Ca (mmol.)	Mg (mmol.)
Ascaris lumbricoides (Rogers, 1945)*	—	6·64	—	119	—	—	—
A. lumbricoides (unpublished data of Hobson, Stephenson & Eden)†	1·017	6·82	0·91	129	24·6	5·9	4·9
Parascaris equorum (Flury, 1912)	—	5·147	0·923	—	—	—	—

Material	Cl (mmol.)	Total P (mmol.)	Glucose (mmol.)	Protein N (mmol.)	Non-protein N (mmol.)	Osmotic pressure as ≡mmol. NaCl	Conductivity as ≡mmol. NaCl
Ascaris lumbricoides (Rogers, 1945)*	55·1	12·0	1·22	495	35·7	—	—
A. lumbricoides (unpublished data of Hobson, Stephenson & Eden)†	52·7	17·04	—	—	—	199	142
Parascaris equorum (Flury, 1912)	62·2	—	—	—	—	—	—
P. equorum (Schopfer, 1924, 1926*a*)	20·5	—	—	—	—	185	—
P. equorum (Duval & Courtois, 1928)	58·2	—	—	—	—	167	—

* Averages calculated from data in Table 1 of Rogers' paper. † Average values.

It was a fairly close correspondence with the results obtained by Collander (1926) with collodion membranes.

that this variation is not merely the result of differences between individual hosts but occurs from time to time within the same animal.

In a series of papers Schopfer (1924, 1925, 1926*a*,*b*, 1927*a*,*b*, 1932) has published the results of his studies of the osmotic relations between intestinal nematodes and cestodes and their hosts. From these and from the data of Vialli (see Schopfer, 1927*a*) he concluded that such species normally live in a slightly hypertonic environment. This conclusion is also supported by Duval & Courtois (1928) and by additional data from our laboratory (unpublished).

Schopfer (1925) studied the change in weight of *P. equorum* exposed to hypotonic (distilled water) and hypertonic (sodium chloride solutions) media, finding that the nematodes gained weight in the former and lost in the latter. Approximate stability of weight was maintained in 1·0–1·1 % solution of sodium chloride (i.e. 171–188 mmol. NaCl). The swelling in hypotonic solutions was confirmed with specimens exposed to diluted contents of the intestine of the horse (Schopfer, 1926*a*,*b*). He noted also (1925) that the curve of the swelling in distilled water attained a maximum which was maintained for several hours and then descended slightly. Further, the curves for individual specimens, even from the same host, varied considerably in their slope and the maximum reached. He explains these facts (1925, 1932) on the hypothesis that the maximum of the swelling curve is determined by the elastic tension of the body wall which offers mechanical resistance to the increase in volume. To this is added the contraction of the muscles, and yet another factor is the amount of fluid in the body of the worm at the beginning of the experiment; the less fluid it contains the more can enter by osmosis. All these factors vary from one individual to another and account for the differences observed.

Panikkar & Sproston (1941), using an unidentified species of *Angusticaecum* from the tortoise (*Testudo graeca*), observed that after the worms had been immersed in tap water for 4 days they were still alive, and that the osmotic pressure of the body fluid was equivalent to that of 1·157–1·280 % NaCl (about 198–219 mmol.). Exposure to diluted or full-strength sea water caused an increase in the osmotic pressure, and the authors conclude that, although the worms 'can maintain a high concentration of body fluid in media containing little or no salt' they possess no mechanism for keeping this fluid hypotonic when they are exposed to an external medium of high osmotic pressure. Experiments with ligatured worms led to the further conclusion that the observed results could not be fully explained by changes in water content but must also involve movements of ions in both directions across the body wall.

Hobson, Stephenson & Beadle (unpublished work) have studied the osmotic relations of *Ascaris lumbricoides* in various dilutions of sea water. Their experiments were carried out with larger numbers of worms than were used by Schopfer, and measurements of osmotic pressure (by the freezing-point method), electrical conductivity (as a measure of the total ionic concentration) and chloride concentrations were made on pooled samples of the body fluid of from sixteen to forty worms. The basic medium used in this work was 30 % sea water, which was the concentration found to cause minimal change in weight of the animals and which was employed as a balanced saline solution. The worms were exposed to 20, 25, 30, 35 and 40 % sea water for periods varying from 40 to 87 hr. It was found that the worms had reached an approximate equilibrium in less than 40 hr., and that no significant alteration in any of the factors measured occurred up to the longest time of exposure used in these experiments.

In the body fluid of worms exposed to 30 % sea water there was little change in the osmotic pressure or conductivity but a rise of about 50 % in the chloride concentration. It is noteworthy, however, that, whereas the osmotic pressure of the body fluid of fresh worms is much less than that of the intestinal fluid of the pig in which they live, the average difference being equivalent to about 70 mmol. NaCl, it is slightly (about 10 mmol. NaCl) greater than that of 30 % sea water. There is, in fact, a reversal of the normal osmotic gradient across the body wall.

The osmotic pressure, conductivity and chloride concentration of the body fluid were almost in direct proportion to those of the external medium within the range of concentrations employed in these experiments. The worms under these conditions are nearly poikilosmotic, as was suspected by Panikkar & Sproston (1941) in the case of *Angusticaecum* in the more concentrated media.

One point, however, requires further mention. The chloride concentration of the body fluid of fresh *Ascaris lumbricoides* is about 52 mmol., and that of the intestinal fluid of the host about 62 mmol. or rather more. When the worms are transferred to 30 % sea water having a chloride concentration of 166 mmol., the body-fluid chloride rises but only to about 78 mmol. This difference in the internal and external concentrations of chloride is maintained throughout the range of dilutions of sea water used. Moreover, it was found that the body wall of worms in a saline solution apparently has the power of adjusting the internal chloride concentration so that it is maintained at a level below that of the external medium. Normal or ligatured worms whose internal chloride has been raised by exposure to 30 % sea water, could, when they were removed to an isotonic saline solution in which half the chloride had been replaced by nitrate, reduce the body-fluid

chloride to a value below that of the external medium. Similar observations were made on eviscerated cylinders of the body filled with body fluid of known chloride content. Thus the worms appear to have the power, when immersed in a saline solution, of maintaining the chloride of the body fluid at a level below that of the external medium in such a way that the ratio of the two concentrations is, within the limits investigated, fairly constant.

In a series of unpublished experiments Hobson & Stephenson exposed *A. lumbricoides* to certain artificial saline media as well as to 30 % sea water. Analyses of the salts of the body fluid and of the artificial media were carried out by Dr Eden.

The results show that in the sea-water medium there is a considerable fall in the potassium and phosphorus contents of the body fluid, while sodium and magnesium are little changed; there may be a slight fall in the calcium; the chloride has already been discussed. With the artificial media the most significant results were that an increase in the potassium concentration led to a corresponding, but not equal, rise in the internal concentration. The magnesium concentration of the body fluid seemed to be independent of that of the medium. The value for phosphorus in the body fluid was not altered by the small amount (2 mmol.) added to the artificial media. It should be noted that the concentration of inorganic phosphorus in sea water does not as a rule exceed 0·003 mg. atoms/l. (Sverdrup, Johnson & Fleming, 1942, p. 182).

Rogers (1945) also followed the changes occurring in the concentration of certain constituents of the body fluid of *A. lumbricoides* kept in a slightly hypotonic saline medium. He found a progressive increase in the chloride content, which was more marked in the absence of phosphate from the medium and which suggested that it might be taking the place of fatty acid excreted. Protein always decreased, but, after about 40 hr., non-protein nitrogen rose markedly. He attributed the first of these effects partly to starvation and the second to 'leakage from cells and the hydrolysis of protein'. If phosphate was present in the medium, both the phosphate content and the copper-reducing power of the body fluid rose steadily, but if phosphate was absent, both tended to fall, although the reducing power increased again after about 70 hr.

(3) *Other factors in the environment which affect the distribution of parasitic nematodes along the alimentary canal of the host*

It is well known that the various species of parasitic nematodes which inhabit the alimentary tract of a host such as the sheep are not distributed along the whole canal. Each has a more or less well-defined zone in which it occurs, although there is overlapping and may even be coincidence.

The factors concerned in determining these specific distributions have not been fully investigated. Tetley (1935*a*) found that the genera *Trichostrongylus*, *Strongyloides*, *Nematodirus* and *Cooperia* are distributed along the intestine of the sheep from the pylorus in the order stated, and that 'the species of any one genus share a common area'. He thought that the site of infection is determined in the larval stage and that the stimuli involved 'arise in the duodenum at the point of entrance of the bile and pancreatic juice. Specific differences in distribution are due to inherent dissimilarities in rates of reaction to the stimuli.'

A study of the species of *Nematodirus* occurring in New Zealand lambs led Tetley (1935*b*) to a similar conclusion. 'It was found that in the cases of both *N. filicollis* and *N. spathiger* the distribution of numbers along the intestine, when plotted, assumed the form of a normal, frequency curve.' Although the areas of distribution were almost identical, the positions of the maximum numbers of the individuals of the two species were distinct and separate. There was little variation with the age of the infection, and Tetley considered that there can have been little movement of the adult worms even within the normal area of infection. Davey (1938*b*) illustrates a similar type of distribution of *Trichostrongylus colubriformis*, *Cooperia curticei* and *Nematodirus* in the sheep. Tetley (1941) measured the lengths of the spicules of specimens of *Cooperia curticei* collected from different parts of the area of distribution in the same individual host and found no significant differences. If the lengths of the spicules can be taken as an indication of the size of the worms, then the whole of this area must be equally favourable for growth.

In view of the considerable fluctuations in the hydrogen-ion concentration which occur from time to time in the alimentary canal of a vertebrate, it might be expected that nematode parasites would not be very sensitive to this environmental factor.

Davey (1938*b*) has investigated the *p*H range which can be tolerated by a number of species of nematodes parasitic in the sheep. The resistance of the worms in buffered saline solutions was determined with the results shown in Table 13. Of these species, *Ostertagia circumcincta* is an inhabitant of the abomasum of the sheep and is therefore normally exposed to a medium of low *p*H value. The species of *Trichostrongylus* examined are usually found in the duodenum, where there is much fluctuation of the hydrogen-ion concentration. Davey points out that their greater resistance to acidity is probably correlated with this, and that both may also occur in the abomasum. He found the remaining species, namely, those of *Nematodirus*, *Cooperia* and *Strongyloides*, are killed relatively

quickly in solutions of high hydrogen-ion concentration. This is in agreement with the fact that the *p*H in the intestine rises with increasing distance from the pylorus.

Whitlock & Leasure (1938) and Whitlock, Link & Leasure (1939) investigated the *p*H range of *Strongylus vulgaris in vitro*. They found it to be *p*H 6·5–8·0 which agrees well with that of the normal environment. An irreversible toxic effect was produced if specimens were exposed to a medium of *p*H 4·0–5·5 for 4 hr. or more.

Table 13. (*Davey*, 1938*b*)

	*p*H
Ostertagia circumcincta	3·2– > 9·0
Trichostrongylus colubriformis	3·6– > 9·0
T. vitrinus	3·6– > 9·0
Nematodirus filicollis	3·9– 8·5
Cooperia oncophora	4·0– > 8·5
Nematodirus spathiger	4·2– 9·0
Cooperia curticei	4·4– > 9·0
Strongyloides papillosus	*c.* 4·6

Davey (1938*b*) also examined the action of bile salts on nematodes parasitic in the alimentary canal of the sheep. He found that *Trichostrongylus colubriformis* and *T. vitrinus* from the duodenum were considerably more resistant than *Ostertagia circumcincta* from the abomasum, or *Cooperia curticei*, *Nematodirus filicollis* and *N. spathiger* from the hinder part of the intestine. Sodium glycocholate seemed to be rather more toxic than sodium taurocholate. He suggested that the greater tolerance of the species of *Trichostrongylus* may be an additional reason why these are abundant near the entrance of the bile duct to the exclusion of less resistant forms.

(4) *The resistance of intestinal nematodes to digestion by the host*

The problem of the resistance of intestinal parasites to digestion by the host has for long attracted attention. Since the most abundant metazoan parasites, the cestodes and nematodes, are covered externally with a cuticular layer which is particularly well developed in the latter group it is, perhaps, natural that this structure should have been regarded as possessing a protective function. It is, in fact, possible that the cuticle does play an important part in preventing digestion by the host and McCoy (1935) assigns to it a major role in this connexion, although he does not deny the part played by anti-enzymes. It must be remembered, however, that a well-developed cuticle is characteristic of all nematodes, and while it has, no doubt, contributed greatly to the success of the group as a whole, it cannot be regarded as a special adaptation to a parasitic mode of life. The observations of Filipjev (1934), already quoted, suggest, however, that the cuticle may be more resistant to the passage of dissolved substance in parasitic than in free-living nematodes. The work of Chitwood (1936) and others referred to previously also indicates that the cortical layer of the cuticle of *Ascaris* is not digested by proteolytic enzymes.

The absence of a cuticular lining in the intestine also indicates that some other protective mechanism must be present. Northrop (1926) called attention to the well-known fact that pepsin and trypsin will not digest living organisms, and concluded from his own and other experiments that the living organism is protected from the action of external enzymes by the cell membranes which do not allow them to penetrate. Burge & Burge (1915) thought that protection from digestion is given by oxidation of the enzyme by the tissues of the parasite. They described experiments in which segments of dead *Ascaris*, normally digested by activated pancreatic juice, were protected by being permeated by nascent oxygen produced by the action of platinum black on hydrogen peroxide.

It is impossible to assess from the relatively short experiments of Northrop and others how potent a factor is this 'natural' resistance of living organisms to digestion by proteolytic enzymes, but there is strong evidence for the existence of another mechanism in nematodes and cestodes.

Weinland (1903*a*, *b*) was the first to suggest that specific anti-enzymes are part of the protective mechanism of intestinal helminths against the digestive juices of their hosts. He found that an aqueous extract of *Ascaris* possessed the power of inactivating trypsin. Dastre & Stassano (1903, 1904) obtained results similar to those of Weinland, but believed that the action was antikinasic rather than antitryptic. Hamill (1906) re-examined the question and proved that the antibody present in extracts of *Ascaris* is an antitrypsin and has no action on enterokinase. He also described other characteristics of the antibody. He found that it is readily destroyed by boiling in alkaline but is resistant in acid solution. He also pointed out that Weinland's observations, confirmed by Dastre & Stassano, that the inhibitory power is destroyed at 100° C. is explained by the fact that in both cases alkaline media were used. Hamill also found that the inhibitory substance dialyses readily, is soluble in 66 % alcohol but is precipitated by 85 % alcohol. He suggested 'that in structure the antibody may prove to be of the same order of complexity as some of the amino-acids'.

According to Sang (1938), Fetterolf (1907) extended Weinland's original observation by finding a similar anti-enzyme in the tapeworm, *Taenia saginata*. This was not confirmed by de Waele (1933), who found that living intact proglottides were unaffected by artificial gastric or pancreatic juice containing

pepsin or pancreatin from the ox. They were similarly resistant to natural human gastric juice and to pancreatin extracted from human pancreas immediately after death. A small cut in the cuticle of the proglottides led, however, in all cases to rapid digestion. He concluded, therefore, that protection against the action of digestive juice is afforded by the impermeable cuticle. De Waele also found that proglottides exposed first to gastric and then to pancreatic juice were digested. He attributed this to the formation, in the second medium, of carbon dioxide which opens minute pores in the cuticle and so allows penetration of the enzyme. Sang (1938) was unable to repeat this observation on *Ascaris*.

Working with lambs, Stewart (1932–3*a*) found that a heavy nematode infestation resulted in the utilization of only 40–60 % of the crude protein of the diet. As the degree of infestation became lower under experimental conditions, so the percentage utilization became greater. If reinfestation occurred the utilization was again reduced. In other experiments Stewart (1932–3*b*) made chloroform water extracts of worms from the abomasum of the sheep and found that these inhibited the proteolytic action of pepsin. He suggested the term 'nezyme' for the type of substance responsible for this action to avoid confusion with those called 'anti-enzymes' by immunologists.

Harned & Nash (1932) described the preparation of antitrypsin from *A. lumbricoides* by a modification of Hamill's (1906) procedure. The worms were frozen, ground and extracted with chloroform water. The extract was centrifuged and alcohol was added to 66 %. It was then filtered and the crude antitrypsin in the filtrate precipitated by increasing the alcohol concentration to 85 % and acidifying to 0·001 *N* by addition of sulphuric acid. The antitrypsin, which is very soluble in water, was purified by dissolving the fractional precipitation with alcohol. It was found that the fractions precipitated by alcohol concentrations lower than 70 % contained a large amount of protease as well as having a considerable antitryptic action. The fraction obtained with 70–80 % alcohol contained a large amount of antitrypsin but a very small quantity of protease. The anti-enzyme preparation had also a slight antipeptic effect. Introduced with insulin into the stomach or duodenum of a depancreatized dog it protected the insulin from digestion and produced a fall of blood sugar. Insulin and antitrypsin introduced into the duodenum of a normal dog had no effect on the blood sugar.

A quantitative study of the action of the anti-enzyme of *A. lumbricoides* was made by Sang (1938). The animals and worms were ground with sand, extracted with chloroform water, and the filtrate was sterilized by passing through a Berkfeld filter. In the later experiments the Berkfeld filtrate was acidified with hydrochloric acid, boiled and filtered again. The extract inhibited the proteolytic action of pepsin and trypsin, but had no such effect on papain. The inhibitory effect was found to become more marked when larger amounts of the extract were used, but after a certain point, further increase resulted in a rise in digestion of protein. Sang's experiments indicated that the proteolytic action of the extract is due to the same substance as that which caused the inhibition of trypsin, and he was unable to confirm the work of Harned & Nash (1932) already quoted, which suggests that these two properties are associated with separate compounds. Sang suggests the name 'ascarase' for this substance and shows that its inhibitory action is produced as the result of combination with the enzyme. It is readily diffusible, precipitated by 70–80 % alcohol and by less than half-saturation with ammonium sulphate and is a substance 'of the order of a primary albumose'. Ascarase was found to be present in all the tissues, the order of abundance in those of the female being ovary, oesophagus, gut, body fluid, ovojector, lateral line, uterus and cuticle (i.e. body wall, since the muscle layer was included). In the male the order of abundance was similar, the gonad containing the largest amount.

Bonsdorff (1939) found that aqueous extracts of the cestodes *Diphyllobothrium latum*, *Taenia saginata* and of the nematode *Ascaris lumbricoides* would inhibit the proteolytic activity of normal human gastric juice on casein at about neutrality, but had no such action on trypsin, pepsin and papain in acid media. On the contrary, the extract of *Diphyllobothrium latum* had itself a proteolytic action which was maximal at about *p*H 4; this property was shared by *Taenia saginata* and *Ascaris lumbricoides*, but to a smaller degree. This author concluded that his experiments disprove the existence of anti-enzymes in these species and that the problem of resistance to digestion is probably more complicated and is connected with the resistance of living tissues to enzymatic influences in general.

Collier (1941), on the other hand, showed that a crude extract of *A. lumbricoides* had a strong inhibitory effect on pepsin and trypsin but none on papain, and that it had no proteolytic activity. He was able to isolate the trypsin inhibitor by fractional precipitation, and stated that it is not precipitated by trichloracetic acid and has the properties of a polypeptide. He agreed with Sang (1938) that the inhibitor probably acts by forming a reversible combination with the trypsin. Collier criticizes the experiments by which Sang demonstrated the proteolytic activity of his *Ascaris* extracts on the grounds that he measured digestion by formol titration which is not specific for proteolysis, and did not allow for the possibility of peptidases being present. Collier considers that the strong proteolytic activity

reported by other workers is probably due to bacterial contamination or to the use of non-specific methods of determination.

The protease-inhibiting substances of parasitic worms are ineffective against enzymes of the papain group. Mendel & Blood (1910) found that the proteolytic action of papain from the latex of the paw-paw (*Carica papaya*) was not inhibited by saline extracts of *Ascaris lumbricoides* which had a strong antipeptic and antitryptic action. Berger & Asenjo (1940) observed the destructive action of solutions of crystalline papain on the tissues of *Ascaris*. Robbins (1930) made from the sap of *Ficus* sp. (probably *F. laurifolia* or *F. glabrata*) a preparation of a proteolytic enzyme resembling trypsin, which he named ficin. It digested living *Ascaris* rapidly, a 0·1–0·2 % solution producing lesions which penetrated the whole thickness of the body wall in 2 hr. These observations were confirmed and extended by Walti (1937, 1938). Berger & Asenjo (1939) obtained similar results with pineapple juice which contains bromelin, another enzyme of the papain group. The same authors (1940) also found that a 0·7 % solution of crystalline papain in M/18 phosphate-phthalate buffer at *p*H 5 would digest *A. lumbricoides* almost completely in 17 hr. These observations are interesting, as both Robbins and Berger & Asenjo point out, since latexes of *Carica papaya* and of *Ficus* spp. have for long been in use as anthelminthics. It appears from these observations that the anti-enzyme of *Ascaris* is not effective against these plant proteases but only the enzymes which it encounters in its normal habitat.

V. THE NUTRITION OF INTESTINAL NEMATODES

(1) *Feeding habits*

It is not necessary to survey in detail the considerable literature dealing with the feeding habits of the various species of intestinal nematodes. It has been well reviewed by Hoeppli (1927), Lapage (1937), Ackert & Whitlock (n.d.) and others.

While there is, of course, no doubt that different species have different methods of obtaining their nourishment, the outstanding question is the relative importance of food material which can be absorbed directly and that which requires digestion by the worms. The many observations which have been made upon the habits of various species and the demonstration, to be referred to presently, of the presence of digestive enzymes in extracts of the alimentary canal of several species form a strong body of evidence in favour of the view that intestinal nematodes do not derive their nourishment entirely from the simple absorption of materials produced by the action of the host's digestive juices or of the constituents of the blood plasma.

The lack of success which has attended the efforts of many investigators to cultivate parasitic nematodes *in vitro*, whether the species used have been inhabitants of the alimentary canal, the blood or other tissues makes it difficult to assess the importance of simple absorption without digestion in the nutrition of these animals. Weinland & Ritter (1902) found that when about 4 % dextrose was added to the saline medium (1 % NaCl) in which *Ascaris lumbricoides* were kept, the consumption of reserve glycogen in certain individuals was decreased. They concluded that the sugar might even be absorbed. There was no similar uptake of laevulose or glycogen. Their results with dextrose were irregular, and in some experiments no evidence of glycogen conservation was obtained. They thought, therefore, that the alimentary canal of the worm must be the site of absorption and not the body wall as in *Taenia*. Hoffmann (1934) performed somewhat similar experiments with *Ascaris lumbricoides* immersed in 1 % NaCl to which 1·5–2 % glucose, fructose or galactose had been added. From direct estimations of the medium after about 6 hr. he concluded that the worms were able to absorb all three sugars to the extent of 0·00061 g. glucose, 0·00055 g. fructose and 0·00080 g. galactose/g. animal/hr. As Hoffmann points out, in Weinland & Ritter's experiments the sugar uptake was not measured directly and the glycogen loss only by difference. Since the same animals could not be used for both glycogen estimations there is here a source of error which may explain the discrepancy. The unpublished data of Hobson, Stephenson & Hobson show that there is a progressive decrease in the glucose concentration of the body fluid of *A. lumbricoides* kept in saline solution (30 % sea water) for several days. This decrease is arrested by the presence of an approximately equal concentration of glucose in the external medium. If the external glucose concentration is raised appreciably that of the body fluid is also increased, although not to so great an extent. This does not, however, occur when worms are ligatured at the anterior and posterior ends, a fact which indicates that glucose is absorbed through the alimentary canal and not through the body wall.

Rogers (1945) found a decrease in the copper-reducing power of the body fluid of worms starved in a saline medium from which phosphate was absent. If phosphate was present there was a steady increase.

It seems probable that some species of parasitic nematodes derive a considerable part of their nourishment by direct absorption of suitable materials dissolved in the fluids surrounding them. Very little is known, however, of the substances which they absorb in this way, apart from the very incomplete observations upon the absorption of sugars by *Ascaris* mentioned above. McCoy (1935)

considers that the Filarioidea, which are parasitic in the blood and other tissues, and the Trichuroidea, which inhabit the alimentary canal or the respiratory or excretory systems, probably depend largely upon the simple absorption of dissolved substances. Hsü (1938) states that certain of the Filarioidea, such as *Dirofilaria immitis*, feed upon cells of blood. Müller (1929) investigated *Trichuris leporis*, *T. suis* and *T. trichura*, and concluded that the nutrition of these species is 'osmotic', the tissues of the host being dissolved by the secretion of the head glands, while the function of the alimentary canal is excretory.

Many of the Strongyloidea live attached to the wall of the alimentary canal of the host, feeding on the mucosa or on blood. *Ancylostoma duodenale* abrades the intestinal mucosa with its teeth and apparently feeds upon the abraded tissue. It also causes haemorrhage which may be very severe. Wells (1931) made direct observations upon the feeding of *A. caninum* by exposing a loop of the intestine in anaesthetized dogs infected with this species of hookworm. He found that the oesophagus made rapid sucking movements at a rate of 120–250 a minute, and calculated that a single worm might remove an average of 0·84 c.c. of blood per day. Nishi (1933) made a similar observation but gave a smaller figure for the amount of blood passing through the worm's anus. Martin & Ross (1934), on the basis of measurements of the phosphorus content of the eggs of *Haemonchus contortus* and on the assumption that this is wholly derived from the blood, estimated that 2000 female worms would need to extract at least 29 c.c. of blood per day even if the whole of the phosphorus available could be utilized. The part played by the blood in the nutrition of worms such as *Ancylostoma caninum* is, however, somewhat uncertain. Wells (1931), for example, pointed out that the blood passed through too quickly to be freely digested. Yamada & Kazuo (1934) and Yamada & Inoue (1934) found no digestion of red and white corpuscles, and thought that the haemorrhage is incidental to the feeding of the worm; they observed, on the other hand, digestion of cells of the mucosa in the intestine of the parasite. Lapage (1937, p. 45) points out that 'no one has yet proved, however, that any of these blood-eating species can digest the blood taken in', and suggests that Davey's (1938*b*) 'failure to keep adult *Ostertagia* alive outside the host either in pure blood or in media containing it, Coutelen's (1928, 1929) failure to cultivate microfilariae and the writer's (1935) to cultivate the first parasitic larvae of Trichostrongylidae in media containing the constituents of blood, suggest that important constituents of the food are not obtained from blood'. Rogers (1940*a*) made spectroscopic examination of the contents of the alimentary canal of *Strongylus edentatus*, *S. vulgaris* and *Syngamus trachea* as well as of fourth-stage larvae of *Strongylus vulgaris*. Haematin was present in every case, and he concluded that all of these species feed upon blood and are able to digest haemoglobin.

It is probable that the Ascaroidea obtain their food from the intestinal contents of their hosts. It has been stated that *Ascaris lumbricoides* and *Parascaris equorum* feed upon blood, but there is no satisfactory evidence that either of them do so regularly although they will take it into the alimentary canal if it is present. Hoeppli (1927) obtained a positive benzidin reaction with the contents of the alimentary canal of both these species, and Galli-Valerio (1935) confirmed this result with *Ascaris lumbricoides*. Rogers (1940*a*) could detect haematin in the intestine of only two out of twenty *A. lumbricoides*. Lièvre (1934), using spectroscopic methods, could not find haemoglobin in the intestine of *A. lumbricoides* or *Parascaris equorum*, but did so in 75 % of the specimens of *Toxocara canis* (= *Belascaris marginata*) which he examined. Rogers (1940) also found haematin in the gut contents of both *T. canis* and *T. mystax*. Mueller (1928) found considerable amounts of blood, which he recognized histologically, in the intestine of *Anisakis dussumierii* (*simplex*).

Ascaris lumbricoides and *Parascaris equorum* are always found free in the intestine of the host, but, as has been indicated above, some workers have thought that they may damage the mucosa. Hoeppli (1927) cites such observations by Greim (1915) with reference to *P. equorum*. He also refers to work by Vogel (apparently unpublished) in which human subjects infected with *Ascaris lumbricoides* were fed with animal charcoal. In the first case the worms subsequently expelled contained no carbon in the intestine, but in the second an appreciable amount was present. Archer & Peterson (1930) gave infected patients a barium meal and later found, by X-ray examination, evidence of ingestion by the worms. Experiments carried out in our laboratory show that specimens of *A. lumbricoides* frequently ingest considerable quantities of powdered charcoal when this is added to the medium in which they are kept.

Li (1933*a*, *b*) investigated the feeding of *A. lumbricoides*, *Toxocara canis*, *Toxascaris leonina*, *Ascaridia galli* (= *lineata*) and *Heterakis gallinae* by feeding the hosts with charcoal, Merck's soluble starch, beef meal and clotted blood. Evidence was obtained that all these species ingested the gut contents of the host although this could only be shown in one out of ten *Ascaris lumbricoides* examined. Li (1933*a*) also found living bacteria in the intestine of a number of species of nematodes. Most of these bacteria belonged to the *coli* group and were similar to those occurring in the host.

The nutrition of the fowl nematode, *Ascaridia galli*, has been studied by Ackert and his collaborators. Ackert (1938) showed that the infective larvae of this species, after hatching from eggs ingested by chickens, lives among the duodenal villi for the first 9 or 10 days and then bores with its anterior end into the mucosa on which it probably feeds. It withdraws into the intestinal lumen 17 or 18 days after hatching, where it reaches maturity about 20 days later.

Ackert and his collaborators studied the growth of *A. galli* in chickens fed normally and in birds nourished by intramuscular injections of glucose solution (Ackert & Whitlock, 1935; Ackert & Freeman, 1936; Ackert, Whitlock & Freeman, 1940). The results obtained showed that there was little difference in the growth rate for the first 18 days, but that, after the worms had withdrawn from the intestinal wall, those in the normally fed chicks grew much faster and were more numerous than those in the birds injected with glucose. This was interpreted as showing that the older parasites feed upon the intestinal contents of the host. Ackert (1938) also found that specimens of *A. galli* 3 weeks or more old could not grow if they were placed in the body cavity of chicks and concluded that this was due to absence of suitable nutriment in this situation.

(2) *Digestion and intestinal absorption*

Investigations of the enzymes of parasitic nematodes have generally been carried out on extracts of the whole body or of the alimentary canal and have usually, as pointed out by Ackert & Whitlock (n.d.) taken no account of the possible effects of bacteria or of the distinction between intracellular and extracellular enzymes.

Kobert (1903) found in various tissue extracts of an ascarid of the dog an amylase which acts upon starch and inulin and also enzymes which split substances such as emulsin, amygdalin, salicin, helicin, arbutin, phloridzin, coniferin, aesculin and quercitrin. Schimmelpfennig (1903) examined the fluid obtained after cutting up the body of *Parascaris equorum* and showed the presence of a diastatic enzyme which could break down raw starch or starch paste to dextrin and sugar. He also found an enzyme similar to trypsin which was most active in alkaline and less so in neutral solution. There was also slight proteolysis in acid solution which he attributed to a pepsin-like enzyme.

Flury (1912) showed that extracts of the alimentary canal of *Ascaris* could digest fibrin, egg albumen, glycogen, starch and various fats. The digestion of carbohydrate was more rapid than that of protein or fat. Sodium chloride extracts of the whole body could, in addition, invert cane sugar and contained an aldehydase.

Abderhalden (1911) reported the presence of proteolytic enzymes in the gut of *Toxocara canis*. Bondouy (1910) demonstrated the presence of a lipase in *Strongylus equinus*. Enigk (1938), by means of feeding experiments carried out *in vitro*, was unable to find a lipase in *Graphidium strigosum*, a species which is parasitic in the stomach of the rabbit. The worms died within 24 hr. if milk or other fats were added to the medium. He showed, however, that fibrin, stained with carmine, is digested in the midgut, but that raw rice starch is not unless it has been previously heated to 60° C. for 30 min. The *p*H of the gut was investigated by adding various indicators to the medium; in the beginning of the midgut it was 4·4–4·8, while in the posterior part and in the hindgut it was somewhat below 7·0.

The most extensive investigation of the digestive enzymes has been made by Rogers (1940*c*, 1941*a*, *b*) on those present in extracts of the alimentary canals of *Strongylus edentatus* and *Ascaris lumbricoides*. In these experiments the oesophagus was removed before the rest of the gut was ground and extracted. In each case an amylase (Rogers, 1940*c*) was present, but they were not identical in the two species. Estimates of the amyloclastic and saccharogenic activities showed these to be maximal at *p*H 8·0 in *Strongylus edentatus* while in *Ascaris lumbricoides* they increased with rising *p*H over the whole range studied (*p*H 5·3–9·4). The effect of various salts on the enzymes was also investigated. It was found that, whereas potassium iodide had the greatest effect in assisting the amyloclastic action of the enzyme from *Strongylus edentatus* it did not activate extracts from *Ascaris lumbricoides*, which were most effectively activated by sodium bicarbonate. Invertase activity of extracts from both species was very slight. Osazone tests on the products of starch digestion showed that dextrosazone was found by the action of extracts of *Strongylus edentatus*. Extracts of *Ascaris lumbricoides* produced no recognizable crystals. Rogers points out that the two amylases must be distinct and that the one present in *A. lumbricoides* cannot be derived from the pancreatic juice of the host. He quotes the statement of Groll (1924) that the optimum *p*H of the pancreatic amylase of the pig is 5·5–6. Rogers also states that 'the situations of the parasites in their hosts are such that the reaction of host juices ingested would give optimum conditions for the action of the parasites' carbohydrate splitting enzymes'. He found that the *p*H of the intestinal contents of *Strongylus edentatus* varied between 7·71 and 8·03, with an average of 7·82. These are considerably higher values than those which Enigk (1938) found in *Graphidium strigosum*, but are in good agreement with the observed *p*H optimum for the amylase *in vitro*. Rogers suggests that the maximal activity of the enzyme of *Ascaris lumbricoides* at *p*H 9·4 is

explained partly by the hydrogen-ion concentration and partly by the presence of bicarbonate in the buffer used.

Rogers (1941*a*) found that extracts of the intestine of both *Strongylus edentatus* and *Ascaris lumbricoides* contain enzymes capable of hydrolysing olive oil and ethyl butyrate. In each case the optimum *p*H was 8·0, but sodium bicarbonate had an activating effect on the enzymes obtained from *A. lumbricoides* but not on those from *Strongylus edentatus*. The action of bile salts revealed a further difference between the two species. Sodium taurocholate retarded fat digestion by the enzymes obtained from both species. Sodium glycocholate, on the other hand, promoted fat-digestion by enzymes obtained from *Ascaris lumbricoides* but retarded it with those from *Strongylus edentatus*. These effects are in accordance with the habits of the worms. *Ascaris lumbricoides* lives upon the contents of the small intestine of the pig, which does not contain taurocholate, while *Strongylus edentatus* feeds upon the mucosa and blood of the large intestine of the horse. The fat-digesting capacity of the two species differed, especially in respect of esterase activity which was about two and a half times as great in *S. edentatus* as in *Ascaris lumbricoides*.

Rogers (1941*b*) also investigated the proteolytic activity of these two species. Using gelatin, blood albumin and casein as substrates he showed the presence of a protease in extracts of the intestine of both species. Judging from the amounts of 'free acidity' and 'formaldehyde acidity' produced, both proteases resembled trypsin but had a maximum activity at *p*H 6·2. *Strongylus edentatus*, which is a tissue feeder, had a much greater capacity for digesting proteins than *Ascaris lumbricoides*. According to Rogers, if the extracts from a given weight of the intestines of the two species were compared '*Strongylus* digested 4·9–8·3 times as much gelatin, 12·5–40·9 times as much casein and 2·5–5·2 times as much blood-albumin as *Ascaris*'. He also studied spectroscopically the course of digestion of haemoglobin by the extracts and found that, in both species, it was most rapid in the more acid media. Oxyhaemoglobin was first reduced and later haematin was found.

Comparatively little attention has so far been paid to the investigation of the parts played by different regions of the alimentary canal of parasitic nematodes in digestion and absorption of food. Apart from the purely mechanical effects produced by the teeth of such species as *Ancylostoma duodenale*, lesions resulting from the external digestion of the mucosa of the host by a number of nematodes have been described (see, for example, Linstow, 1907; Hoeppli, 1927; Feng, 1931; Schuurmans Stekhoven & Botman, 1932). The general conclusion reached is that these lesions are caused by the secretions of the oesophageal glands. Hsü (1933*a*) has reviewed the literature dealing with these glands and has also (1933*b*) given descriptions of their structure in nineteen species belonging to the Ascaroidea. He concludes that they are responsible for external digestion by species such as *Anisakis*, in which they are well developed, but that in others they do not produce liquefaction of the host's tissues, only inflammation. While there is no doubt that many species such as *Contracoecum rectangulum* (Linstow, 1907), *Physaloptera clausa* (Feng, 1931) and *Proleptus obtusus* (Schuurmans Stekhoven & Botman, 1932) do produce histolysis of the tissues surrounding the head of the nematode and that this is due to extra-intestinal digestion, there is little direct evidence that the secretions of the oesophageal glands are responsible, although this is highly probable. Hoeppli & Feng (1931) failed to obtain any digestion *in vitro* of tissue or egg albumen by means of extracts of the oesophagus of *Physaloptera clausa*. Chitwood (1938*a*), however, found a protease in a saline extract of the oesophagus of *Ascaris lumbricoides*. Acting on buffered calcium chloride milk, it gave maximum digestion at *p*H 5·2 and 8·4 and none at *p*H 8, which he assumed to be the isoelectric point of the enzyme. Rogers (1940*c*) could not find an amylase in extracts of the oesophagus of *Strongylus edentatus*, but he did not investigate the possibility of the presence of other enzymes.

Almost nothing is known of the sequence of events in the intestine of parasitic nematodes. The observations of Enigk, previously referred to, that the *p*H of the anterior part of the midgut in *Graphidium strigosum* is markedly less than that of the more posterior part and of the hind gut, suggests a functional difference between these regions. Rogers (1940*c*) noted that the amylolytic activity of extracts of the intestine of *Strongylus edentatus* was somewhat less when these were made from the posterior than from the anterior half of this organ.

The absorption of iron saccharate by the intestine of *Ascaris lumbricoides* has been studied by Hirsch & Bretschneider (1937*b*). They found that, after starving the worms in Tyrode's solution for 6 days, iron saccharate added to the medium could be detected in the lumen of the gut within a few minutes, and after only 10 min. free iron could be demonstrated in the intestinal cells. Enigk (1938) fed rabbits infected with *Graphidium strigosum* with powdered wood and animal charcoal. Ten days later the rabbits were killed and the worms were examined. In cells of the first part of the midgut no carbon particles were present; in the second to third fifths there were a few just below the bacillary layer (Stäbchensaum); in the fourth part the cells were greyish black, and in the terminal region only a few particles were found. Similar results were obtained

with trypan blue. It seems, therefore, that the midgut cells can ingest solid particles, and that, perhaps, intracellular digestion may also take place.

VI. THE METABOLISM OF PARASITIC NEMATODES

(1) *Introduction*

The metabolism of parasitic nematodes has been the subject of investigation for more than 40 years, and, in many of its aspects, it is still not fully understood. The available evidence suggests strongly that, *in vitro*, and probably also in the normal environment, the ordinary energy requirements are fulfilled by the consumption of the carbohydrates which form so large a proportion of the body. Fat is present in considerable amount but comparatively little is known of its metabolism. The data on nitrogen metabolism are still more scanty, due, probably, to the fact that, so far, it has been impossible to keep intestinal nematodes alive apart from their hosts for more than a short time, within which the only striking changes are connected with the carbohydrate content.

(2) *Nitrogen metabolism*

The little that we know about the nitrogen metabolism is derived from the general analyses of the body and from the evidence for the presence of proteolytic enzymes, already summarized in this review. We have also a very small amount of information about the nitrogenous excretory products.

Flury (1912) failed to demonstrate the presence of uric acid or creatinine in the body of *Ascaris lumbricoides*, but he found ammonia, amines and substances giving the biuret reaction in the external medium when he kept these worms in a minimal volume of saline solution. Weinland (1901*b*), who used the same species, found in two experiments that 11 and 15 mg. N were excreted per 100 g. worm per day. These figures were based on only two determinations, but in a later paper (1904*b*) he reported more extensive experiments which gave the slightly higher values of 15–20 mg. N excreted per 100 g. worm per day. He found no evidence for the production of free nitrogen and concluded that this element is excreted mostly in the form of ammonia and ammonia derivatives (about $\frac{1}{3}$ total N as ammonia) and not as nitrite or nitrate. Brand (1934) estimated that 100 g. *A. lumbricoides* eliminates 30 mg. N in 24 hr., of which 10 mg. is in the form of eggs and 20 mg. as dissolved material.

Except for the observations of Weinland and Brand just mentioned, little attention has been paid to the nature of the nitrogenous excretory products of nematodes. Chitwood (1938*a*), however, countered the claim of Mueller (1928) that the so-called excretory system of *A. lumbricoides* is not really excretory in function by collecting fluid from the excretory pore in a capillary tube and testing with xanthydrol. Crystals were formed similar to those of dixanthyl urea developed under similar conditions when urea solutions were used. Chitwood estimated by comparison that the concentration of urea is about 0·02 % in the excretory fluid of fresh worms. When the worms were kept in normal saline the concentration gradually fell and the test was negative after 24 hr. He suggests, therefore, that the urea is not produced by the worm but is derived from the host and excreted. In his brief note he does not give any further reasons for this interpretation of his observations.

(3) *Fat metabolism*

There is some uncertainty as to the part played by fats in the metabolism of parasitic nematodes, although these compounds are conspicuous among the apparent reserve substances of these worms. To some extent this is due to the difficulty of interpreting the results of histological methods for the staining of 'fats', but few chemical estimations have been made.

Weinland (1901*b*) made two determinations, by means of ether extracts, of the fat content of fresh specimens of *A. lumbricoides*, and found 1·46 and 1·51 % of fat in the total body weight.

Flury (1912) made a more extensive examination of the fats and fat-like substances of fresh *A. lumbricoides*. His principal results are summarized in Tables 14 and 15.

Table 14. Ascaris lumbricoides (*Flury*, 1912)

	Percentage of dry substance	Percentage of fresh worm
Total fat	10·88	1·630
Non-volatile fat	7·00	1·319
Volatile fatty acids and other volatile substances	3·88	0·311

The substances extracted with ether and chloroform consisted of free fatty acids, neutral fats, lecithins, aldehydes, esters and small amounts of other, mostly volatile, compounds. Glycerol was present only in small quantities, and cholesterol could generally not be detected.

Flury also reported the presence of a hitherto unknown compound which he called ascaryl alcohol and to which he assigned the formula $C_{32}H_{64}O_4$. He thought that this substance might take the place of alcohols, glycerol and cholesterol in the body of the nematode. Fauré-Fremiet (1913*b*, *d*) discovered ascaryl alcohol independently, and almost simultaneously and gave a fuller account of its properties. According to him it is a white substance, melting at about 82° C. and crystallizing as masses of radially arranged, fine needles. It is an acid alcohol and

forms water-soluble soaps with alkalies. An acetyl derivative was prepared and elementary analyses by Leroux indicated the formula $C_{20}H_{40}O_2$ for the ascarylic acid and $C_{20}H_{39}O_2(CO_2CH_3)$ for the acetyl compound. Flury obtained ascaryl alcohol from extracts of the whole body of the worm, but Fauré-Fremiet found that it was confined to the female reproductive system. He stated that in the oocytes it forms droplets or crystals about 4–5 μ long which had been originally described by von Beneden as 'corps réfringents'. Fauré-Fremiet suggested that in the cells it might be in the form of an ether. It is expelled from the cytoplasm after fertilization, and some of it seems to be used to form the inner membrane or chorion of the egg. According to Fauré-Fremiet the material extracted by acetone makes up about 24 % of the dry weight of the egg and yields, after saponification, ascaryl alcohol.

Table 15. *Analysis of* 100 *g.* '*fats*' *of* Ascaris lumbricoides (*Flury*, 1912)

	g.
Total fats	65·03
Volatile fatty acids	31·07
Saturated fatty acids	30·89
Unsaturated fatty acids	34·14
Non-volatile fatty substances	68·93
Unsaponifiable fatty substances	24·72
Glycerin	2·40
Lecithin	6·61
Lipoids of unknown constitution	1·24
Acid value of the fats	25·10
Iodine value of the fats	57·80
Iodine value of the unsaturated fatty acids	75·20

Regarding the fatty acids present, Flury states that he had isolated and identified oleic, stearic and a small amount of palmitic acid. The volatile lower fatty acids were represented by valerianic and butyric with smaller quantities of prunic, propionic and acrylic acids.

Brand made several measurements of the fat content of fresh *A. lumbricoides*. Extractions with petrol ether (1934) gave values of 1·17 and 1·18 % of the wet weight. In a later paper (1941) he describes experiments in which, using the method of Kumagawa & Suto (1908), he obtained the value of $1{\cdot}75 \pm 0{\cdot}07$ %.

Brand (1938*b*) also examined the fat content of larvae of a species of *Eustrongylides* from the fish, *Fundulus heteroclitus*. Ether extractions yielded amounts averaging 1·1 % of the weight of the fresh worms.

These limited data seem, therefore, to indicate that the storage of fats in parasitic nematodes is not very great. The amount seems to lie between about 1·0 and 1·8 % of the total body weight.

The distribution of fat in the various tissues has been investigated by a number of workers. Flury (1912), using chemical methods, found it, in *Ascaris lumbricoides*, to be most abundant in the reproductive organs of which 4·0–6·25 % of the wet weight and 11–24·2 % of the dry weight was composed of fat. The body wall also contained fat to the extent of 1·00 % of its wet weight and 3·87 % of its dry weight. He gave no figures for the alimentary canal, but stated that the greatest part of the fat is included in the reproductive organs and body wall.

Kemnitz (1912) investigated the distribution of fat in the same species by histological methods. He concluded that the hypodermis is the principal site for the storage of fat. Large amounts of it are present in the dorsal, ventral and lateral lines. The musculo-epithelial cells contain only a small quantity of fat, which is confined to the non-contractile parts.

Unlike Flury, Kemnitz found that the ovaries and testes contained no fat. In the intestine he demonstrated fat with some difficulty and found it only in a certain number of the cells. He could not detect fat in the cells of the oesophagus.

Fauré-Fremiet (1913*a*) found neutral fats staining with osmium tetroxide in the intestinal cells of *Parascaris equorum*. He also described the presence of fats in spermatogonia, oogonia and young oocytes. He concluded from the results of his microchemical tests that the fat is a triglyceride containing oleic acid.

The occurrence of fat in the intestinal cells seems to be widespread in nematodes parasitic in both animals and plants and also in free-living species. In addition to those previously quoted, the papers of Davaine (1857) and Perez (1866), cited by Goodey (1930), Payne (1923), Peters (1927), Goodey (1930), Giovannola (1936), Hirsch & Bretschneider (1937*a*) and Rogers (1939, 1940*d*) may be mentioned.

As fat is usually regarded as a reserve source of energy it might be expected that, under the conditions of starvation usually imposed upon parasitic nematodes when they are kept *in vitro*, it would disappear rapidly. This, however, does not seem to be the case. Weinland (1901*b*) estimated the fat content of *Ascaris lumbricoides* kept in 1 % NaCl solution for 4 and 5 days, and obtained values of 1·24 and 1·45 % of the body weight respectively. These he compared with values of 1·46 and 1·51 % for the fresh worms and pointed out that the value of 1·24 % was too low because the worms swelled during the course of the experiment. He concluded that there was no appreciable consumption of fat by the starving nematodes.

Brand (1934) found 1·17 and 1·15 % of fat in *A. lumbricoides* after 24 hr. starvation under aerobic and anaerobic conditions respectively. Comparing these values with 1·18 % fat found in fresh worms he came to the same conclusion as Weinland. In a later paper (1941) Brand describes more extensive and careful experiments in which individual female

specimens of *A. lumbricoides* were weighed and then starved for 5 days in 1 % saline at 37° C. At the end of this period they were analysed for fat by the method of Kumagawa & Suto (1908). The eggs from each individual were collected by centrifugation and analysed by the same method. The fresh worms contained $1{\cdot}75 \pm 0{\cdot}07$ % fat, and starved worms $1{\cdot}67 \pm 0{\cdot}05$ %, while the fat extracted from the eggs corresponded to $0{\cdot}05 \pm 0{\cdot}013$ % of the weight of the worms. He concluded that, since the fat content of the starved worms together with that of the discharged eggs is almost equal to that of the fresh animals, there is here no evidence that, under these conditions, there was any appreciable consumption of fat for the purpose of energy production.

Enigk (1938) attempted unsuccessfully to cultivate the ensheathed third larva of *Graphidium strigosum* which is parasitic in the stomach of the rabbit. Staining showed the presence of both glycogen and fat in the cells of the midgut. In the fasting larvae the glycogen became greatly reduced, but there was no apparent change in the fat content. Enigk suggests that the fat is a useless by-product of the metabolism which he assumed to be of the anoxybiotic type.

There is, however, evidence that fats do play an important part in the physiology of parasitic nematodes.

Fauré-Fremiet (1913*d*), in his description of the development of the gametes of *Parascaris equorum*, states that fat droplets can be demonstrated histologically in oogonia and spermatogonia and in young oocytes and spermatocytes. In the oocytes the fat soon disappears, and this process coincides with the appearance of glycogen. Fat reappears in the fertilized egg as soon as the chorion is formed, but its origin is unknown. Fauré-Fremiet suggests that it might be derived from the ascaryl ether which vanishes at this time and might be saponified with release of glycerol and fatty acids; or it might be formed from the lipoid sheaths of the droplets containing calcium phosphate which are present in the oocytes.

Hirsch & Bretschneider (1937*a*) described the changes which take place in the intestinal cells of *Ascaris* kept in Tyrode's solution or in plain salt solutions at 37° C. for 6 days. The proportion of cells containing fat fell from 73 % in the fresh worms to about 20 % on the first and second day of starvation. On the third day it increased to 90–94 %, and after this fell until on the sixth day only 38 % of the cells contained fat. They also noted that the amount of fat stained by osmium tetroxide in the individual cells did not show a marked decrease during the first 2 days, although the number of fat-containing cells was much reduced. By the third day there was a great shrinkage in the size of the masses of fat in the cells. This shrinkage continued until, on the fifth and sixth days, only small fat droplets were present in the cells. They concluded, not only that fat was used by the worms but also that the increase in number of fat-containing cells after 3 days' starvation implies either that fat is formed from the glycogen or that it has been redistributed. They considered the former to be the more likely.

Mueller (1928) also presented evidence for the ability of the tissues of *A. lumbricoides* to consume fat. He found it possible to 'culture' small pieces of the lateral line, which is one of the main sites of fat storage, in hanging drops for as long as 8 days. At the end of this period the pieces were fixed in Fleming, sectioned and stained in Safranin O and Lichtgrün. The fat, as well as the glycogen, had completely disappeared, although the tissue showed every sign of being alive at the time of fixation.

There is some evidence that fat is utilized during development. Dyrdowska (1931) found that eggs of *Parascaris equorum* incubated in an atmosphere of nitrogen at 38° C. showed after 2 days a decrease in their content of stainable fat. Giovannola (1936), using the histological technique described by Goodey (1930), examined the fat content of the larvae of *Necator americanus*, *Ancylostoma caninum*, *Nippostrongylus muris* and *Strongylus fülleborni*. He was able to support the view of Payne (1923) and Cort (1925) that the quantity of fat granules in filariform hookworm larvae is an index of 'physiological age'. He found considerable variations in the fat content at different stages of the life history. The amount was greatest in the adults, but smaller maxima were observed in the unsegmented eggs and in the filariform larvae. He concluded that fat forms what he terms the 'food reserve' which he distinguished from the 'energy reserve' of glycogen. The former is, he thinks, accumulated during periods of the life history which precede times during which feeding is difficult or impossible. Thus, fat is stored in the adult, the eggs and during the long migration of the larvae. Glycogen forms the 'energy reserve' for immediate usage. Rogers also found that the amount of stainable fat in infective larvae of *Ancylostoma caninum* (1939) and *Haemonchus contortus* (1940*d*) for some weeks cultivated in the laboratory decreased with age.

The view that fat is not normally utilized by intestinal nematodes for energy production rests mainly upon the failure of Weinland (1901*a*) and Brand (1941) to detect any loss of fat in starving adult specimens. Schulte (1917), indeed, found that the fat content of *Ascaris lumbricoides* actually increases under anaerobic conditions by about 0·1 g./100 g. body weight in 24 hr. He considered it probable that this fat was formed from carbohydrate. It must be remembered, however, that the period of starvation was in all cases comparatively short. Judging by the figures given by Weinland (1901*b*)

and Brand (1937*a*) for the glycogen consumption of *A. lumbricoides* as well as those obtained by Toryu (1935) with *Parascaris equorum*, in no case have the worms been starved sufficiently long to exhaust their reserves of carbohydrates. Whether fat may or may not be expended by starving nematodes for energy production can only be decided by experiments involving a longer period of starvation.

It has also been objected that the utilization of fat as a source of energy demands the presence of oxygen. Although some workers consider that the normal metabolism of *Ascaris* is completely anaerobic, this cannot be considered as finally proved. This question is discussed more fully in another section of this review.

That fats play a more important part in the physiology of nematodes than was attributed to them by Enigk (1938) cannot be doubted. Flury (1912) discovered ascaryl alcohol in the body of *Ascaris*, and Fauré-Fremiet (1913*b*, *d*) reported its presence in the female reproductive organs. The fact that this compound must have been synthesized by the worms supports the view, as Brand points out (1941), that there is a considerable fat metabolism in these animals. The histological changes in the distribution and amount of stainable fat in the intestinal cells of *Ascaris* described by Hirsch & Bretschneider (1937*a*), the disappearance of fat as well as glycogen from Mueller's 'cultured' pieces of lateral-line tissue and the considerable changes in fat content at various stages in the life history of the species of nematodes reported by Giovannola (1936) as well as the observations made by Rogers (1939, 1940*d*), all argue against the view that fat plays merely a passive role. How it is built up, whether from carbohydrate, as suggested by Hirsch & Bretschneider (1937*a*), or by addition of CH_2 groups, from fatty acids, such as valeric, produced during a period of anaerobiosis, as suggested by Mueller (1928) or by some other means, can only be decided by further work. It should be remembered, also, that Rogers (1941*a*) has shown the presence of lipase in extracts from the intestine of *Strongylus edentatus* and *Ascaris lumbricoides*. It is reasonable, therefore, to surmise that since there is a mechanism for breaking down fats these substances are utilized in some way.

On the whole the evidence seems to justify the view that fat present in parasitic nematodes is a true reserve substance, but that it is not normally utilized as a source of energy by the adult intestinal parasite owing to the low oxygen tension usually present in the environment, and the availability of large amounts of glycogen which is more easily broken down under these conditions. If sufficient oxygen is available, at certain stages of the life history, the fat is almost certainly broken down. It is quite possible that this may also occur with the adult worms under aerobic conditions, especially if the glycogen reserve has been exhausted, but this has not yet been proved.

(4) *Carbohydrate metabolism and the problem of respiration*

Michael Foster (1865) discovered more than 80 years ago that *A. lumbricoides*, obtained from the pig, contains a large amount of glycogen. Since then further observations have been made by various investigators on this and other species by means of both chemical and histological methods. These all confirm the view that glycogen is a normal reserve material in adult nematodes parasitic in animals, and is, in many cases, stored in large quantities.

Some investigations have been made on the chemical nature of the glycogen. Weinland (1901*a*) obtained from *A. lumbricoides* a glycogen which had a specific rotation of 189°, and found that, after inversion, it yielded glucosazone. He concluded that it was a polysaccharide of dextrose. Campbell (1936, 1937) isolated from the same species a polysaccharide which, even after removal of all detectable nitrogen, possessed antigenic properties. These were not destroyed by boiling in neutral or acid solution or by digestion with proteolytic enzymes, but were abolished by boiling in alkaline solution. Baldwin & King (1942) pointed out that the chemical data given by Campbell indicate that his polysaccharide was probably glycogen. They also prepared from *A. lumbricoides* a glycogen which 'consists entirely of glucose units and does not differ significantly from other glycogens of the widely distributed 12-unit type'.

The available quantitative data on the total glycogen contents of parasitic nematodes are summarized in Table 16.

It will be seen that the percentage of glycogen is, in every instance, considerable, although there is, as might be expected, a good deal of variation from one species to another and even within the same species. Some of this variation is probably due to differences in the method of estimation. Foster's (1865) value of 2·2 % glycogen in the total body weight of *A. lumbricoides* was obtained by estimating the sugar produced by digestion with saliva and is probably too low. Some of the figures given by Smorodincev & Bebešin (1936) for females of the same species are very high. They do not give the dry weight of the animals, but assuming this to be about 20 % of the total body weight (see Weinland, 1901*b*; Flury, 1912; Schulte, 1917, quoted in Table 5 of this review), maximum and average figures of about 58 and 43 % respectively of glycogen in the dry substance are obtained for the females.

It must, however, be noted that Brand (1937*a*) found that the glycogen content of *A. lumbricoides* which he examined in America was distinctly

less than that of those with which he had worked in Denmark. The values he obtained for the latter (Brand, 1934) were approximately the same as those found by Weinland (1901*a*, *b*) in Germany. Brand suggested that such variations might be accounted for by differences in the feeding of the hosts or by different treatment when they were slaughtered.

If the determinations quoted in Table 16 are taken at their face value without considering possible inaccuracies of method or variations due to sex or origin of sample, the figures given in Table 17 may, perhaps, be accepted as approximate estimates of the average glycogen contents of adult worms of the species which have been investigated from this point of view.

It must be remembered that these figures are by no means of equal reliability, since many more estimations have been made on *A. lumbricoides* and *Parascaris equorum* than on any other species. For the remaining four species no data are available to indicate the degree of variation in glycogen content. The table does, however, give some idea of the inter-specific differences which occur.

The distribution of glycogen in the tissues of adult nematodes has been described in greater or less detail by various authors. ***Ascaris lumbricoides*** and *Parascaris equorum* are, however, the only forms which

Table 16. *Glycogen content of fresh worms*

Material	Glycogen as percentage of Wet weight	Glycogen as percentage of Dry weight	Author
Ascaris lumbricoides	2·2	—	Foster (1865)
	4·2–7·1, av. 5·5	20–34	Weinland (1901*a*)
	5·3 (autumn and winter), 5·5 (summer)	—	Weinland (1901*b*)
	6·77, 6·08, 6·58, 5·89	—	Weinland & Ritter (1902)
	3·650	24·40	Flury (1912)
	5·29–7·18	—	Brand (1934)
	Females: 4·85–11·48; av. 8·67 Males: 2·93–8·42; av. 6·13	—	Smorodincev & Bebešin (1936)
	Females: 3·08–7·80; av. 5·29 ± 0·28 Males: 3·18–8·85; av. 5·77 ± 0·35	—	Brand (1937*a*)
	3·36–6·40	—	Brand (1937*b*)
Parascaris equorum	1·3836, 2·1375	6·918, 10·6875	Schimmelpfennig (1903)
	Females: 2·400–4·664; av. 3·747 Males: 2·220–3·502; av. 2·917	Females: 20·020–25·781; av. 23·068 Males: 13·321–17·980; av. 15·283	Toryu (1933)
	Females: 3·152 Males: 2·746 (each value the average for 5 specimens)	—	Toryu (1935)
	3·332	—	Toryu (1936*a*)
	Females: 3·8 Males: 2·9 (each value the average of 2 determinations)	—	Smorodincev & Bebešin (1936)
Toxocara mystax	4·4	—	Weinland (1901*a*)
	4·4	—	Smorodincev & Bebešin (1936)
Ascaridia galli	Female: 4·63 Male: 3·60	—	Reid (1944)
Strongylus vulgaris	3·5	—	Toryu (1933)
Ancylostoma caninum	*c.* 1·5	—	Brand & Otto (1938)
Ancylostoma duodenale larvae	Nil	—	Busch (1905)
Necator americana larvae	Nil	—	Payne (1923)
Eustrongylides ignotus larvae	8·45 (av. figure)	—	Brand & Simpson (1944)
Filaria equina	2·4, 2·0	—	Toryu (1933)

have been investigated fully. Only isolated observations upon other species are available.

Using histological methods, Busch (1905) showed the presence of glycogen in the muscle cells of *Ascaris lumbricoides*, *Parascaris equorum*, *Enterobius vermicularis*, *Oxyuris vermicularis*, *Strongylus equinus* (*Sclerostomum armatum*) and *Ancylostoma duodenale*, and found that it is almost or quite confined to the non-contractile regions. This confirmed the previous observations of Brault & Loeper (1904). Kemnitz (1912) and Toryu (1933) also found glycogen in the non-contractile parts of the muscles of *Ascaris lumbricoides* and *Parascaris equorum*. Brand (1938*b*) describes a similar distribution of glycogen in the muscle cells of the larva of a species of *Eustrongylides* parasitic in the tissues of the fish *Fundulus heteroclitus* and subsequently identified as *E. ignotus* (Brand & Simpson, 1944).

Table 17

Species	Glycogen content of adult worms as percentage of total body weight
Ascaris lumbricoides	5·6
Parascaris equorum	2·9
Toxocara mystax	4·4
Ascaridia galli	5·5
Strongylus vulgaris	3·5
Ancylostoma caninum	1·5
Filaria equina	2·2

In the cells of the intestine Brault & Loeper (1904) failed to find glycogen, but its presence in this tissue has been demonstrated by many later workers, for example Busch (1905), Kemnitz (1912) and Hirsch & Bretschneider (1937*a*) for *Ascaris lumbricoides*, and Busch (1905), Fauré-Fremiet (1913*d*) and Toryu (1933) for *Parascaris equorum*. Brand (1938*b*) found large amounts of glycogen in the intestinal cells of the larval *Eustrongylides* referred to above, and Enigk (1938) mentions its occurrence in this situation in larvae of *Graphidium strigosum*.

Glycogen is also deposited in the hypodermis and in the dorsal, ventral and lateral lines. Busch (1905) notes that there is relatively little in the median lines, and Kemnitz (1912) states that it is absent from the phagocytic cells and the cuticle.

Before dealing with the distribution and history of glycogen in the reproductive system, reference must be made to the small number of instances in which chemical analyses have been made on the two sexes. The results of these have been included in Table 16, but they are somewhat contradictory.

Toryu (1933, 1935) obtained distinctly higher figures for the glycogen content of females than of male *Parascaris equorum*. A similar difference was found by Smorodincev & Bebešin (1936) both for this species and for *Ascaris lumbricoides*, and by Reid (1944) for *Ascaridia galli*. Brand (1937*a*), on the other hand, states that in *Ascaris lumbricoides* analysed as soon as possible after removal from the host the glycogen content did not differ greatly in the two sexes. In the males, the average value was $5{\cdot}77 \pm 0{\cdot}35$ % and in the females $5{\cdot}29 \pm 0{\cdot}28$ %. He pointed out that this difference is not significant, but that it appears that males do not, at any rate, store less glycogen than females. Possibly they contain a little more, since, in fifteen out of twenty determinations, each made on a batch of ten worms, the males gave the higher figure. In this connexion the results of Toryu's (1933) analyses of the glycogen contents of the individual organs of male and females *Parascaris equorum* should be considered. The most important of these are summarized in Table 18. His evidence shows that from 22 to 41 % of the total glycogen of the female worm is present in the reproductive system while the corresponding system in the male contains only about 0·3–2·3 %. There is no apparent sexual difference in the glycogen contents of the other tissues. In his analyses of whole female worms he found great individual variations (2·400–4·664 % of wet weight), but his results seem to suggest, as he himself points out, that the glycogen content is greater in large than in small individuals. Presumably, this is correlated with the development of the reproductive system. In males the individual variation was less (2·220–3·417 % of the wet weight), and no correlation with size could be detected.

Although the data of Toryu are insufficient to give final proof, because of the great individual variation which undoubtedly exists, they indicate that in *P. equorum*, at least, the males and females do not differ greatly in their capacity for storing glycogen in the non-reproductive tissues. Any sexual difference in the total glycogen content, therefore, is to be attributed mainly to the degree of development and physiological condition of the reproductive system and to the ratio of the body wall and alimentary canal to the body as a whole. Whether *Ascaris lumbricoides* differs in this respect, as Brand's results seem to suggest, cannot be decided from the present data. The whole question cannot be settled until much more information is available about the relative growth of the organs of nematodes and about their glycogen contents.

Busch (1905) showed the presence of glycogen in the ovary of both *A. lumbricoides* and *Parascaris equorum* by means of staining methods and noted that it seemed to be most abundant in the unripe eggs. Kemnitz (1912) confirmed these observations on *P. equorum*. He found that, in the upper part of the female reproductive system, glycogen occurred in the cells of the walls but not in the oogonia. Lower down it was distributed equally between the parietal epithelium and the oogonia, while still farther down it was confined to the oogonia. Fauré-Fremiet (1913*d*) gave a more

detailed description of the changes which take place during the development of the eggs of *P. equorum*. He stated that young oocytes contain fat but little or no glycogen. The fat then disappears and, at the same time, glycogen is laid down. He suggested that in view of this coincidence the fat is converted into glycogen. From analyses of the whole ovary he obtained an average glycogen content of 21 % of the dry weight. Assuming on the basis of Flury's (1912) figures (see Table 6) that the dry weight of the ovary is approximately 25 % of the wet weight, the glycogen content amounts to about 5·25 % of the total weight of this organ, a figure which agrees quite clearly present in the fertilized egg in considerable amounts.

Toryu (1933) also employed both histological and chemical methods on *P. equorum*, and his results are in general agreement with those of Fauré-Fremiet. By staining he found large amounts of glycogen in the oogonia and oocytes but only a small quantity in the eggs in the uterus. The results of his chemical analyses are given in Table 18. The average glycogen content of the ovary is about 6·5 % and that of the uterus 1·6 %.

In the male of *Ascaris lumbricoides* and *Parascaris equorum*, Busch (1905) reported the presence of

Table 18. *Glycogen contents of tissues of* Parascaris equorum (*data from Toryu*, 1933)

	Muscle		Intestine		Ovary		Uterus		♀ reproductive system	
Sex and wt. of worm	Glycogen percentage of wet weight	Glycogen content as percentage of total glycogen	Glycogen percentage of wet weight	Glycogen content as percentage of total glycogen	Glycogen percentage of wet weight	Glycogen content as percentage of total glycogen	Glycogen percentage of wet weight	Glycogen content as percentage of total glycogen	Glycogen percentage of wet weight	Glycogen content as percentage of total glycogen
♀, 3·8 g.	4·783	64·436	0·322	1·288	4·546	24·161	1·345 (1·53)	10·116	—	—
♀, 3·92 g.	5·750	53·772 (64·28)	0·645	1·908 (2·28)	6·537	20·861 (24·95)	1·495	7·100 (8·49)	—	—
♀, 4·0 g.	7·910	71·129 (72·23)	1·269	2·967	7·416 (6·66)	15·072 (13·76)	2·369	10·833 (11·00)	—	—
♀, 4·9 g.	5·431	75·522	0·599	2·256	4·516	13·523 (12·75)	1·605	9·636	—	—
♀, 6·8 g.	5·126 (4·71)	56·593	0·435	1·923	9·463	35·840	1·095	5·644	—	—
♂, 1·37 g.	4·446	95·807	0·427	1·864	—	—	—	—	0·781 (0·47)	2·327
♂, 1·39 g.	4·951	97·577	0·623	2·157	—	—	—	—	0·063	0·266
♂, 1·49 g.	5·297	96·168	0·695	2·340	—	—	—	—	0·695 (0·61)	1·427

Notes (1) In the above table 'Muscle' includes the whole body wall, i.e. cuticle, hypodermis and muscle cells. 'Intestine' includes oesophagus, mid- and hindguts.

(2) This table contains many arithmetical errors. The more serious of these are shown by the figures in brackets which are recalculated from the values given in Toryu's original Table 5 for the weight of tissue and glycogen content (omitted here for reasons of space).

well with the results of Toryu (1933, see Table 18 of this review). Fauré-Fremiet points out that his value is probably too low for the mature oocytes, since the material which he used included the parietal epithelium as well as part of the germinal zone, the oocytes and oogonia of which contain little or no glycogen. In the fertilized eggs the glycogen content was found to average 4·67 % of the dry weight or only about one-fifth of the concentration in the ovary. Part of this loss he assumed to be represented by the glucosamines of the egg shell, but estimates of these showed that a quantity of glycogen equal to 7–9 % of the dry weight was still unaccounted for. This, he suggested, might have been converted into lower fatty acids such as butyric and valerianic acids which, according to him, are

glycogen in both the developing gametes and the fully formed spermatozoa. Kemnitz (1912) found that the distribution of glycogen in the reproductive system of the male was generally similar to that in the female, except that there was none in the spermatocytes although it was present in the spermatids. His histological observations were confirmed by Fauré-Fremiet (1913*a*) and later by Toryu (1933), who gives, in addition, figures which show that the concentration of glycogen in the reproductive system is much smaller in the male than it is in the female and is, apparently, of the same order of magnitude as in the intestine.

Glycogen is consumed whether oxygen is present or not. Weinland (1901*b*) measured the rate of consumption in *Ascaris lumbricoides* under aerobic

conditions and found that, on the average, 0·7 g. glycogen was used by 100 g. worms in 24 hr. This amount was slightly increased if oxygen, hydrogen or carbon dioxide was passed through the medium. Schulte (1917), using the same species, obtained the figure of 1·1 g. glycogen consumed per 100 g. worms per day when the animals were kept in 0·9 % NaCl saturated with carbon dioxide and also containing oxygen. Brand (1934) found that they consumed 1·39 g./100 g./day under anaerobic conditions and 0·85 g. when oxygen was present. He noted (1936) that, in the absence of oxygen, the glycogen consumption decreased after the first day. Brand (1937*a*) also found that there was a sexual difference. In the female the anaerobic glycogen consumption per 24 hr. for the first 3 days averaged 1·32, 1·06 and 0·67 g./100 g. worm respectively. For the male the corresponding figures were rather higher, being 1·36, 1·59 and 1·00 g. He explained this on the ground that, under experimental conditions, the females are relatively inactive after the first day, whereas the males become quiet only during the third day. Ro (1939), working with the same species, also found, under anaerobic conditions, a higher rate of glycogen consumption by the males than by the females.

Toryu (1935, 1936*b*) found a similar falling off in the glycogen consumption of *Parascaris equorum* after the first day and noted that more is used by the males than the females. Under anaerobic conditions the average consumption for the first 24 hr. was, in the case of the males, about 1·32 g./100 g. and, for the second 24 hr. 0·73 g.; with the females the corresponding figures were 1·97 and 0·18 g. Whether the apparent difference in the glycogen consumption of the males and females of the two species is significant can only be decided by further work. The number of worms used by Toryu in his experiments was small.

Reid (1944) found that starvation of the host produced a rapid fall in the glycogen content of the fowl nematode, *Ascaridia galli*. In worms obtained from hosts fed normally the average values were 4·63 % glycogen for females and 3·60 % for males. Starvation of the host for 24 and 48 hr. resulted in the reduction of these contents to 1·35 and 0·49 % respectively in the females and to 1·36 and 0·46 % in the males. 48–96 hr. starvation caused the nematodes to be passed out of the hosts.

Brand & Simpson (1944) give figures for the carbohydrate consumption by larvae of *Eustrongylides ignotus* under experimental conditions. These larvae are tissue parasites of the fish *Fundulus heteroclitus*.

Some histological investigations have been made on the changes in the glycogen content of starving worms. Kemnitz (1912) found that glycogen decreased most rapidly in the intestinal epithelium, from which it had completely disappeared after the first day. He explained this on the grounds that the intestinal epithelium may be regarded merely as an organ for the temporary storage (Übergangsstation) of glycogen. From the contractile part of the muscle cells glycogen disappeared after 1–2 days, while it remained in the non-contractile part until the death of the animal (5–6 days). The other tissues showed a continuous but not so rapid decrease in the glycogen content.

Weinland & Ritter (1902) examined the effect on the glycogen content of *Ascaris lumbricoides* produced by adding dextrose, laevulose or glycogen to the medium in which the worms were kept. A positive result was obtained only with dextrose, which in two instances caused conservation, i.e. a smaller reduction in the total content of glycogen after 2 days. Negative results were obtained by addition of boiled starch and dextrin to the medium. More striking effects were produced by the injection of small quantities (up to 0·5 c.c.) of strong solutions (usually 30–50 %) of various sugars into the worms. An actual increase in the glycogen content was observed in one case after injection with dextrose; in other cases conservation occurred. Weinland & Ritter concluded that dextrose could be used to build up glycogen, and that laevulose and, possibly, galactose cause conservation of glycogen. Of the disaccharides, maltose resulted in conservation, but lactose had no effect. Kemnitz (1912), following Weinland & Ritter's procedure, injected 40 % dextrose into *A. lumbricoides* on two successive days and found histological evidence of a marked increase in the amount of glycogen deposited, especially in the intestinal epithelium, the lateral lines and the hypodermis. Brand & Simpson (1944) found that the consumption of reserve glycogen by the larvae of *Eustrongylides ignotus* was reduced if glucose was present in the medium.

Krummacher (1918) attempted to measure the heat production of *Ascaris lumbricoides*, but his results were inconclusive. Meier (1931) was successful, but confined his experiments to starving animals in which he found a heat production of 0·30 cal./g. body weight/hr. Hoffmann (1934) extended these observations to specimens immersed in a nutrient medium composed of 1 % NaCl + 1·5–2 % glucose, fructose or galactose. He obtained the following mean values for heat production per g. body weight per hour:

Glucose	0·50 cal.
Fructose	0·47 cal.
Galactose	0·29 cal.

He also made direct measurements of the sugar removed from the medium per g. body weight per hour (mean values):

Glucose	0·00061 g.
Fructose	0·00055 g.
Galactose	0·00080 g.

He pointed out that, not only do these experiments show that monosaccharides can be taken up by the worms but that they are also used for energy production since the heat production is greater than that of starving worms as judged by Meier's (1931) results. Hoffmann, himself, does not seem to have made any determinations on starving worms.

Brand (1936, 1937*b*) found that *A. lumbricoides* can, under aerobic conditions, resynthesize part of the glycogen consumed during a preceding anaerobic period. After 20 hr. anaerobiosis one-twentieth to one-tenth of the glycogen broken down was reformed when oxygen was admitted. This, as he points out, is a much smaller proportion than found in vertebrate muscles in which four-fifths to five-sixths of the broken-down glycogen is resynthesized. He suggests that this difference may be explained by the fact that *Ascaris* can excrete the end-products which might otherwise be used in this way. There is no evidence to show whether the glycogen is resynthesized from lactic acid or from fatty acids.

Toryu (1935) found that in starving females of *Parascaris equorum* the glycogen granules disappeared from the muscle cells and the hypodermis in about 30 hr. under aerobic and about 48 hr. under anaerobic conditions. Similar results were obtained with the males except that, when oxygen was present, a small amount of glycogen remained up to the point of death, perhaps because they do not survive so long. In the females the glycogen disappears from the oogonia but not from the oocytes and ova. In both sexes the small amount of glycogen in the intestinal epithelium quickly disappears.

The cytological work of Hirsch & Bretschneider (1937*a*) has already been referred to in connexion with fat metabolism. They observed that the large masses of glycogen in the intestinal cells of *Ascaris* begin to break up on the first day of starvation and that only granules can be seen on the second day. These gradually disappear, beginning with those at the base of the cell and proceeding apically.

The presence of haemoglobin in the body fluid or tissues has been recorded in a number of species, e.g. *Dioctophyme renale* (Adducco, 1889; Janicki, 1939), *Ascaris lumbricoides* (Flury, 1912; Keilin, 1925; Krüger, 1936), *Parascaris equorum* (Schimmelpfennig, 1903; Flury, 1912; Fauré-Fremiet, 1913*a*), a larval *Eustrongylides* from the tissues of the fish, *Fundulus heteroclitus* (Brand, 1938*b*), larvae of *Trichinella spiralis* (Stannard, McCoy & Latchford, 1938), and *Camallanus trispinosus* from a turtle (Wharton, 1938, 1941). Cytochrome was discovered in *Ascaris lumbricoides* by Keilin (1925) and by Wharton (1941) in *Camallanus trispinosus*.

Some workers have expressed the opinion that haemoglobin, present in the body fluid or tissues of a nematode, is derived from the host (e.g. Schimmelpfennig, 1903; Fauré-Fremiet, 1913*a*; Schwartz, 1921, cited by Lapage, 1937), but various observations indicate that the pigments are not identical. Adducco (1889), for example, found it impossible to obtain complete reduction of the haemoglobin of *Dioctophyme renale* by means of a vacuum, although it could be accomplished by the use of a chemical reducing agent. Keilin (1925) noted that 'The position of the (absorption) bands in the body fluid of *Ascaris* is approximately intermediate between that of the pig's blood and the HbO_2 in the integument of the worm'. Brand (1938*b*) concluded that the haemoglobin in the larval *Eustrongylides* which he studied is a true constituent of the body, since it was still present after the worms had been kept alive in a haemoglobin-free medium for 1–2 months. Wharton (1941) examined the dissociation curve of the oxyhaemoglobin of *Camallanus trispinosus* and found it to be situated considerably to the left of that of the oxyhaemoglobin of the host, showing that the pigment can take up oxygen at very low tensions. *C. trispinosus* could reduce its own haemoglobin, and Wharton considered that it could take up oxygen from the blood of the host.

Davenport (1945) examined the haemoglobins of the body wall and the body fluid of *Ascaris lumbricoides*. He found that absorption spectra of the oxyhaemoglobins are unusual in that the width and intensity of the α band is distinctly less than that of the β band. The absorption spectrum of the reduced haemoglobin is of the normal type. Neither of the oxyhaemoglobins could be completely deoxygenated *in vacuo* at 20° C. Total removal of oxygen could be brought about by means of sodium hyposulphite *in vacuo* at 8° C. The rate of deoxygenation of the body wall haemoglobin was 10,000 times and of the body fluid haemoglobin 2500 times slower than that of sheep haemoglobin. He concluded that the *Ascaris* body-wall pigment 'may have significance as an oxygen carrier at the low tensions of the gas which prevail in the mammalian gut'.

The widespread occurrence of haemoglobin in parasitic nematodes and the fact that it has been shown in several species to differ from that of the host is strong evidence for the existence of an aerobic metabolism in these animals even if it plays only a minor role. As Davey (1938*a*) points out: 'In any event, it would be remarkable if the nematodes of the alimentary canal synthesized haemoglobin and made no use of it.' The presence of cytochrome in at least two species also supports this view.

The discovery by Wells (1931) and others that hookworms suck far more blood than they digest, and that this is arterial blood, has given rise to the suggestion, not supported by Wells himself, that oxygen may be obtained by these parasites from the haemoglobin of the host. Wells's observations were made on *Ancylostoma caninum*, and Brand &

Jahn (n.d.) calculate that the amount of oxygen theoretically available in the volume of blood passed through the worm in 24 hr. is about ten times that found by Harwood & Brown (1933) to be utilized by this species under experimental conditions.

In attempts to keep intestinal nematodes alive *in vitro* a number of workers have investigated the effects of the presence or absence of oxygen on the survival time. Bunge (1883) found that *Toxocara mystax* could live and remain active for as long as 15 days and generally for 8–10 days in the presence of air; under conditions of very low oxygen tension (he does not claim that oxygen was completely absent) they lived 5–7 days. He repeated his experiments (1889) at low oxygen tensions with other ascarids and stated that *Ascaris lumbricoides* survived 5–7 days. Weinland (1901*b*) was somewhat less successful in keeping *A. lumbricoides* alive. With air or oxygen passed through the same medium as that used by Bunge (1 % NaCl) the worms lived only 3–5 days, and a similar result was obtained with hydrogen. Saturation of the medium with carbon dioxide kept them alive for 7–9 days, but this favourable effect was explained by the high carbon dioxide content of the normal environment. Slater (1925) repeated the experiments of Weinland on the survival of *A. lumbricoides* in a medium through which hydrogen was passed. He says that the worms became 'dormant', thus confirming Bunge's observations. Bunge, however, stated (1889) that *Toxocara mystax* and other ascarids remain alive and in almost continuous movement in the absence of oxygen for 4–5 days. Slater kept his worms in continuous movement by means of periodic electrical stimulation and found that those kept in a medium through which air was bubbled lived somewhat longer than those kept in a medium through which hydrogen was passed. He concludes: 'from these experiments it appears reasonable to conclude that the worms are incapable of continuous movement in the absence of oxygen or its equivalent, and that it is only by avoiding movement that they are able to live for long periods in atmospheres of inert gases.' While this conclusion may be correct, it is unfortunate that the evidence is not more extensive. Only four experiments were performed, each involving six worms, two in air and two pairs in hydrogen. In view of the great variation to be found in the behaviour of these animals, even when they are taken from the same batch and kept under the same conditions, it is desirable that these experiments should be repeated. It should also be noted that the 'dormant' condition observed by Slater is possibly an indication that the worms were in an unhealthy state. Davey (1938*a*) states that '*Ascaris* is an exasperatingly inert creature, and its large size is the only good quality it can be said to have for respiration experiments'. Observations made in our laboratory show that healthy worms under aerobic conditions are far from inert even after many days. Any conclusions drawn from animals which do not display, if not continuous, then at least periodic activity, should be regarded with reserve.

On the other hand, Toryu (1935) found that *Parascaris equorum* survived somewhat longer under anaerobic conditions than if oxygen was present. At 38° C. the differences were not great; in air the average times of survival were 34 and 30 hr. and without air 44 and 35 hr. for males and females respectively. At room temperature (16–19° C.) the difference was more marked, the corresponding figures being 116 and 119 hr. in the presence of air and 161 and 167 hr. in its absence. When he compared the effects of a medium saturated with oxygen with one saturated with carbon dioxide he found that the survival time was much greater in the latter than in the former. The males survived 30 hr. in a medium saturated with oxygen and 74 hr. in one saturated with carbon dioxide. The corresponding periods of survival of the females were 28 and 86 hr. He concluded that the presence of oxygen shortens the life of the worms, but that its concentration is not important. On the other hand, he considered that carbon dioxide is distinctly beneficial. These experiments of Toryu require repetition. The small number of worms, their variability and the very short time for which they survived *in vitro* make it doubtful whether the differences in the survival times which have been mentioned are significant.

Adam (1932) observed that *Ascaris lumbricoides* in closed Petri dishes soon became motionless, but that movement began again as soon as the dishes were opened. He concluded that oxygen has no unfavourable effect.

While the evidence quoted above indicates that forms such as *A. lumbricoides* can withstand long periods of anaerobiosis, the results of Davey (1938*a*) with a number of species of nematodes from the sheep show that this may not be generally true. He found no movement in any of the species tested after about 4 hr. of anaerobiosis. Removal of aerobic conditions caused no recovery after 24 hr. anaerobiosis in *Ostertagia circumcincta*, *Trichostrongylus colubriformis* and *T. vitrinus* and *Cooperia curticei*. *Cooperia oncophora* and *Nematodirus filicollis* were more resistant, recovering after 48 hr. exposure to anaerobic conditions. By keeping *Ostertagia circumcincta* and the species of *Trichostrongylus* in a continually changing medium he showed that death was not due to the accumulation of excretory products in the environment.

It is evident that the relation of intestinal nematodes to aerobic and anaerobic conditions varies considerably. The hookworms which suck blood and are, although they live in the intestine of the host, essentially tissue parasites, may well be mainly

aerobic in their metabolism. This is the opinion of Lapage (1937) and is also supported by the high oxygen consumption per unit of body weight of *Ancylostoma caninum* found by Harwood & Brown (1933), which they state to be more than ten times that of *Ascaris lumbricoides*. Davey's (1938*a*) experiments showing the relatively high degree of intolerance of sheep nematodes to absence of oxygen are somewhat difficult to understand in view of the low oxygen tensions which have been found in the alimentary canal (see Tables 2 and 3). They may, perhaps, be explained by the fact that haemoglobin is apparently present, although this is not specially stated, and by the small size of the worms (Brand, 1938*a*) and the consequently high surface-volume ratio.

The disappearance of glycogen from the tissues of normal or starving worms may be due to its conversion into some other reserve substance as well as to its breakdown for the purpose of providing energy. The first of these possibilities has been suggested by several authors. Fauré-Fremiet (1913*d*) noted that, whereas the glycogen content of the whole ovary of *Parascaris equorum* was about 21 % of the dry weight, that of the fertilized eggs averaged 4·67 %. He thought that part of the glycogen which has disappeared had been converted into glucosamines in the egg shell; the remainder, about 7–9 % of the dry weight, he considered had been converted into lower fatty acids which are, he says, clearly present in the fertilized egg. Hirsch & Bretschneider (1937*a*) considered that some of the glycogen in the gut cells of starving *Ascaris* was turned into fat. Schulte (1917) found an increase of about 0·1 g. fat/100 g. fresh material per day in *A. lumbricoides*, and concluded that it was probably formed from carbohydrate.

There is no doubt, however, that by far the greater part of the glycogen which disappears from nematodes under experimental conditions is utilized for the provision of energy. While there is some doubt about the importance of an oxidative breakdown of glycogen in the metabolism of such nematodes as *A. lumbricoides* it is generally agreed that the principal energy-producing mechanism must be one which is able to work in the absence of oxygen. The details of this mechanism have not yet been fully elucidated.

Bunge (1889) found that *A. lumbricoides* produced, under 'anaerobic' conditions, a considerable amount of carbon dioxide and also a volatile acid which he was unable to identify owing to lack of material. Weinland (1901*b*) measured the carbon dioxide production in the same species and found that it was higher when air was bubbled through the medium than when hydrogen was passed through. The rate of carbon dioxide production when hydrogen passed through the medium was 0·38–0·4 g./100 g. worm/day. Weinland also identified the volatile acid as valeric and gave its rate of production as 0·30 g./100 g. worm/day. He realized the possibility of fatty acid being produced by bacterial action, but concluded that it was formed by the worm because its characteristic smell did not develop when the 1 % NaCl solution which has been used for washing the animals was enriched with dextrose and incubated at 28° C. In a later paper (1904*a*) Weinland describes his preparation of calcium salts of the fatty acids distilled from the medium in which the worms had been kept and concluded that there was a mixture of valeric and caproic with, possibly, other lower fatty acids. In order to investigate further the possibility of fatty acid production being due to bacterial action Weinland (1902) ground the worms with sand and Kieselguhr and then made an extract with 1 % NaCl. He attempted to sterilize this extract by the addition to it of various antiseptics ($As_2O_3 + K_2CO_3$, NaF, $CHCl_3$ and toluol). This extract had a higher carbon dioxide production than the same amount of the whole tissue of the worm and also produced valeric acid.

Schimmelpfennig (1903), working with *Parascaris equorum*, considered that the whole series of volatile fatty acids was probably present. He identified butyric, formic, acetic, caproic, caprylic, propionic and valeric acids. Flury (1912) found free fatty acids and their esters in the saline medium in which worms had been kept. He also found in extracts of the body of *Ascaris lumbricoides* a considerable amount of the volatile lower fatty acids, mainly valeric and butyric, with smaller quantities of formic, propionic and acrylic acids. Schulte (1917) estimated the valeric acid production of *A. lumbricoides* kept in 1 % NaCl saturated with carbon dioxide in sealed bottles. In two experiments he found 1·35 and 0·20 g. of valeric acid respectively per 100 g. worm per day.

Fischer (1924) measured the lactic and phosphoric acid production of *Parascaris equorum* by analysis of the saline medium in which the worms were kept and by autolysis experiments performed with a 'broth' made by grinding up whole worms with sand and incubating under toluol. He also made similar estimations with 'muscle broth' made from the body wall and gut and 'genital broth' from the reproductive organs. As the result of the analysis of the medium surrounding living worms, he found that lactic acid formed about 10 % of the total acidity; only one estimation of phosphoric acid was made. The lactic acid concentration did not increase with the length of time the worms were kept in the solution. Incubation of the broth derived from the whole worms showed that maximum acidity was reached in about 50 hr., and this, calculated as lactic acid, averaged 1·7 %. The further experiments in which broth was made from the body wall and from

the reproductive organs showed that the lactic and phosphoric acid contents approximately accounted for the total acidity. It is interesting to note that, as Fischer pointed out, the reproductive organs produced more lactic and phosphoric acids than the muscles. This may, perhaps, be partly explained by the presence of cuticle in the muscle broth, but it is still remarkable in view of the fact that much glycogen is stored in the hypodermis and muscles and that the latter may reasonably be looked upon as a main site of energy production. Fischer concluded that, while in living animals lactic acid forms only about 10 % of the total acidity, it increases rapidly after death. He also considered the production of valeric acid to be quite unproved and very unlikely.

Brand (1934), working with *Ascaris lumbricoides*, found that the material excreted by 100 g. worms in 24 hr. under anaerobic conditions included 0·71 g. CO_2, 0·22 g. valeric acid and 0·02 g. lactic acid; in the presence of air the total figures were 0·82 g. CO_2, 0·16 g. valeric acid and 0·01 g. lactic acid. Waechter (1934) made ether extracts of the acidified saline medium in which worms had been kept and showed the presence of valeric acid by means of boiling-point determinations and elementary analyses. There was also, perhaps, a small amount of caproic acid. A copper salt was prepared and the crystals proved to be those of *ortho-*, not *iso-*valeric acid. Krüger (1936) measured the production of fatty acid as well as that of carbon dioxide by *A. lumbricoides*. He found the former to be greater on the first day under both aerobic and anaerobic conditions; in the presence of oxygen it decreased continuously, but the anaerobic production remained fairly constant after the second day. He attempted to identify the fatty acids by fractionation, elementary analysis and melting-point determinations. He found valeric acid—probably active valeric acid (methyl-ethyl-acetic acid) in a mixture of *dextro-* and *laevo-*forms. Caproic acid was also present, not the normal acid, but one of the other seven isomers with a branched carbon chain.

Toryu (1936*a*) examined the fatty acid production of *Parascaris equorum* and found that valeric and lactic acids with a trace of propionic acid were formed under both aerobic and anaerobic conditions. The valeric acid production was slightly less in the presence of oxygen, but in each case continued at a steady rate of about 0·3 g./100 g. worm/12 hr. Lactic acid formation did not differ in aerobic and anaerobic conditions but ceased after the first 24 hr. From his histological observations Toryu concluded that the lactic acid was the result of the breakdown of the glycogen in the non-contractile part of the muscle cells, while the fatty acid was produced by the carbohydrate metabolism of all the tissues.

Oesterlin (1938) investigated the fatty acids present in the medium in which *Ascaris lumbricoides* had been kept. The medium was changed every 4 hr. to reduce bacterial action. It was treated with strong alkali and, after sufficient had been collected, it was acidified with sulphuric acid, extracted with ether and fractionated. Formic, acetic and *n*-valeric acids were identified with certainty. Butyric acid was probably present and there were, as well, small amounts of higher acids which could not be identified. The main excretory product was valeric acid.

The preceding account shows that the majority of workers who have investigated the subject are agreed that fatty acids are normal excretory products of *A. lumbricoides* and *Parascaris equorum*. A mixture of acids seems to be present, of which valeric is the most abundant, but others such as formic, acetic, propionic, butyric and caproic have been identified with more or less certainty by various authors. Fischer (1924), on the other hand, considered that the formation of valeric acid by the worms had not been proved and thought that lactic acid was the end-product of carbohydrate metabolism. He found that the lactic acid accounted for only 10 % of the total acidity in the medium surrounding the worms, but he did not investigate the remaining 90 % except to record in one experiment a small amount of phosphoric acid. In autolysis experiments lactic and phosphoric acids accounted for practically the whole of the acid present. His results are thus in conflict with those of Weinland (1902). Fischer does not mention any precautions taken to avoid loss of volatile acids in his experiments and estimations. Brand & Jahn (n.d.) consider that the results of his autolysis experiments are due to differences in experimental conditions influencing the course of the chemical reactions. Slater (1925, 1928), although he made no determinations of the acid production of *Ascaris lumbricoides*, strongly supported Fischer's conclusions. He pointed out that no worker has been able to eliminate bacterial action, and he observed that bacteria from the fluid in which the worms had lived, cultured on a medium containing salt, peptone and glucose, produced a mixture of saturated fatty acids (acetic, butyric, etc.) similar to that found when the animals are kept under experimental conditions. Brand & Jahn (n.d.), however, criticized Slater's conclusions on the grounds that he gave no evidence that the bacteria were sufficiently abundant to produce the amount of fatty acids observed in the experiments with nematodes mentioned above, nor did he explain what substrate could have been used by the bacteria.

While there is no doubt that carbon dioxide is a normal excretory product of such species as *A. lumbricoides* under both aerobic and anaerobic conditions, there have been differences of opinion with regard to the consumption of oxygen, if this occurs.

Weinland (1901*b*) assumed the whole of the carbohydrate metabolism to be anaerobic and explained the carbon dioxide production on the basis of his suggested mechanism for the fermentative breakdown of glycogen yielding valeric acid, hydrogen and carbon dioxide. Many later workers agreed with him, and, although Fischer (1924) raised objections to the fermentation process and Slater (1925) showed that nematodes stimulated to continuous activity lived longer in the presence of oxygen, it was not until 1932 that Adam showed by actual measurements that oxygen is taken up by *A. lumbricoides*. He also found that the rate of oxygen consumption is not affected by a previous period of anaerobiosis and that carbon monoxide has an inhibitory effect upon it. He found that a homogeneous 'broth' prepared by grinding up the muscular layer of the body wall would also take up oxygen for a considerable time.

Harwood & Brown (1933) found that female *A. lumbricoides* consume 0·03–0·45 ml. oxygen at N.T.P./g. body weight/hr. Male specimens used 0·05–0·07 ml., while *Ancylostoma caninum* took up more than ten times as much per g. as the female *Ascaris*. These authors did not measure the carbon dioxide output.

Toryu (1934, 1936*b*) found that the total amount of carbon dioxide produced by *Parascaris equorum* was greater under aerobic than anaerobic conditions. Light decreased the carbon dioxide production although it had no effect on the oxygen consumption. The rate of carbon dioxide formation pursued a characteristic course during the experiment. For the first 2–4 hr. or even longer (up to 12 hr.) little or none was evolved. Then came a period during which carbon dioxide was produced. This was only temporary, and for the last few hours before death, which, in Toryu's experiments, usually occurred after 30–40 hr. or sometimes longer, no carbon dioxide was formed. This course was followed whether the conditions were aerobic or anaerobic. Worms placed in oxygenated Ringer in sealed vessels took up no oxygen during this initial period during which no carbon dioxide was produced. Then oxygen consumption began and continued, although at a somewhat irregular rate, until the concentration in the medium had fallen to about 0·03 vol. %. The R.Q. was about 3 in large females, but much lower, varying around unity, in small females and in males.

Harnisch (1933) experimented with pieces, about 1 cm. long, taken from the anterior third of the body of *Ascaris lumbricoides*. Movement of such pieces was reduced to a minimum. He also used the material obtained by pressing finely chopped pieces through bolting silk. His experiments were carried out at about 28° C. When the pieces were kept in air the oxygen consumption, although it fluctuated considerably, gradually decreased towards the end of the experiment (duration 68 hr. or less). Lower oxygen tensions (11, 6 and 0·8 % were used) caused a reduction in the oxygen consumption which, however, rose again when the pieces were returned to air. Exposure to 95 % oxygen caused, at first, an increase in oxygen consumption to about three times the value found in air. This was not maintained but decreased gradually. Return to air caused a fall in the initial value observed at this oxygen tension followed by a decreased rate of uptake. The oxygen consumption of press-juice in different oxygen tensions resembled, in general, that of the pieces. With the pieces, but not with the press-juice, an oxygen debt was found after exposure to low oxygen tensions. It did not seem, however, to bear any regular relation to the degree of lowering of oxygen tension or to its duration. The carbon dioxide production was fairly constant and did not seem to be connected with the oxygen tension and, consequently, Harnisch concluded that the R.Q. = 0.

In a later paper Harnisch (1935) investigated the oxygen consumption of the gut, uterus, ovary and also that of the body wall from the anterior third of the body, excluding the phagocytic cells and, as far as possible, the lateral lines. An appreciable oxygen consumption occurred which, although extremely variable, was approximately the same for the body wall, uterus and ovary but much greater for the gut. Harnisch also found (1937) that the body fluid consumes oxygen. Tissue preparations free from cells and suspended in sodium chloride solution do not, with the exception of those from the genital organs which take up a small amount. Tissue preparations suspended in body fluid consume a greatly increased amount of oxygen due, he concludes, to the presence of an oxydase in the body fluid.

It should be noted that none of Harnisch's experiments on *Ascaris* was conducted at the normal temperature of the worm's environment or even at the same temperature throughout the series of investigations. In his 1933 paper the temperature is given as about 33° C., in that of 1935 as varying between 25 and 30° C. and in that of 1937 as about 24° C. In the last paper he justifies the use of 24° C. on the grounds that *Ascaris* (which he was comparing with the cestode *Moniezia expansa* from the sheep and certain fish parasites) can remain alive for some time at room temperature. A further point to be taken into consideration is that Harnisch (1933, 1935) mentions that he kept his worms for 2 days at about 40° C. before his experiments. Other workers (see a later section of this paper) have shown that this temperature is harmful and Harnisch does not, in any case, seem to appreciate the importance of accurate temperature control.

Krüger (1936) investigated the respiratory exchange of *Ascaris lumbricoides* by means of a respirometer in which the worms were not immersed in

fluid, but were kept in an atmosphere saturated in water vapour, so that carbon dioxide was not lost by solution. Observations were carried out over a period of about 4 days. The oxygen consumption was greater at first, but decreased later. He found no evidence of the formation of an oxygen debt even when the worms were kept in oxygen-free nitrogen for 48 hr. The carbon dioxide production was also greatest at first and decreased later, although it varied greatly. The R.Q. was initially about 2·6 but decreased to about 1·15 on the second day and later to 1·0, at which it remained constant. In oxygen-free nitrogen the carbon dioxide production was greatly decreased but was not abolished, and it was found that the presence of even 0·5 % oxygen had a marked effect in increasing the output of carbon dioxide. In fact, Krüger (1936, 1937) found a close dependence of both oxygen consumption and carbon dioxide output upon oxygen tension, even up to 100 % oxygen, although the increase was greater in the former than in the latter.

Krüger (1936) found that the oxygen uptake of a small *Ascaris* weighing 1·4 g. was about 78 cu.mm./g./30 min. The oxygen consumption of specimens larger than this, although extremely variable, agreed approximately with the formula

$$O_2 \text{ consumption (cu.mm./30 min.)} = (\text{body weight} - 1{\cdot}4) \times 20 + 110.$$

In a later paper Krüger (1940) showed that the relation between weight and surface fits in well with the Meeh formula

$$O = K \sqrt[3]{g^2},$$

where K has the value 13·96. He concluded that the oxygen uptake is, within certain limits, constant, and thought that the explanation of this lies, not in differing conditions of diffusion or in changes in the proportions of the respiring tissues, but in a decrease in respiratory activity with increasing size.

Krüger (1936) also measured the oxygen consumption of isolated tissues of *A. lumbricoides*. Like Harnisch (1935) he found that all of them took up oxygen, but he differed in finding the uptake of the body wall much greater than that of the other tissues. This part of the body was, in fact, the only one whose oxygen consumption approached that of the intact animal.

From the evidence summarized above it is clear that such intestinal nematodes as *A. lumbricoides* and *Parascaris equorum* as well as their isolated tissues can take up oxygen from their surroundings. This is in disagreement with the view of Weinland, who thought that the metabolism of these worms is essentially anaerobic. Slater (1925, 1928), on the other hand, considered that they are essentially aerobic, and thought that, although they can survive for a long time in the absence of oxygen, they can only do so by reducing their metabolism rate to a very low level and becoming almost motionless. It is worth noting that Stannard *et al.* (1938) found that mature larvae of *Trichinella spiralis*, released from their cysts, are active in the presence of oxygen but motionless if respiration is inhibited by cyanide or lack of oxygen. Slater (1928) endeavoured to show that the carbohydrate metabolism of *Ascaris lumbricoides* agrees with the glycogen-lactic acid cycle worked out for free-living animals, and he argued against the existence of the fermentative type of breakdown suggested by Weinland and others. In support of his view he pointed out that the possibility that bacteria are responsible for the reproduction of fatty acid has not been eliminated, and quoted the discovery by Fischer (1924) of lactic acid as an excretory product and the assertion of the same worker that the formation of valeric acid by the worm is impossible. The evidence that the whole of the fatty acid observed under experimental conditions is bacterial in origin is, as Brand & Jahn (n.d.) pointed out, unsatisfactory.

Another attempt to correlate the metabolism of intestinal nematodes with that of free-living animals has been made by Harnisch (1937). In free-living invertebrates he distinguished two kinds of oxybiotic processes: (1) the normal oxybiosis of animals living under favourable respiratory conditions which is largely independent of the oxygen tension of the medium, and (2) the increased respiration succeeding previous anaerobiosis whose magnitude depends upon the oxygen tension. He considers that the oxygen consumption of *Ascaris* corresponds to the second type of process, and that it is concerned with the oxidation of some substances produced in the course of anaerobic metabolism.

The experiments of Krüger (1936) led him to disagree with the conclusion of Harnisch (1933) that the carbon dioxide production of *Ascaris* is independent of the oxygen consumption and that the R.Q. is therefore zero. Krüger found that fresh specimens have a high respiratory quotient which falls to about 1 during the first 2 days and then remains constant. The carbon dioxide production-oxygen tension curve is a straight line. He also found that when specimens which had a steady oxygen consumption and carbon dioxide production and R.Q. of 0·96 were transferred to 6 % glucose for 25 hr., they showed an increased oxygen consumption and carbon dioxide production and R.Q. of 1·3. Similar results were also obtained by keeping the worms overnight in 3–5 % peptone. He considered that fasting worms can make use of oxygen for energy production, but that it is doubtful whether this occurs under natural conditions.

Laser (1944) has made a more detailed examination of the oxidative metabolism of *A. lumbricoides* (= *A. suis*). Using small worms of not more than

4 g. wet weight in his experiments he found that the oxygen consumption depends upon the oxygen tension. In air it was about 80 μl./g. wet weight/hr., which is in general agreement with the results of previous workers. In 5 % oxygen, however, it was about half and in pure oxygen about four times this rate. The R.Q. was 1·1–1·2 in air and 0·5–0·6 in oxygen. An oxygen debt was developed after anaerobiosis for a period of 17–20 hr.

The oxygen uptake of muscle pulp rose with the addition of methylene blue. It rose still further if the methylene blue was accompanied by sodium succinate. Neither cytochrome *c* nor riboflavine caused a rise, perhaps because they are unable to enter the cells.

A factor which tends to depress the oxygen consumption of *Ascaris* is its apparent inability to destroy oxaloacetic acid, which inhibits succinic dehydrogenase (Das, 1937). Keilin & Hartree (1940) showed that co-enzyme 1 inhibits the cytochrome-succinic system due to the formation of this substance. Addition of methylene blue + succinate to normal *Ascaris* muscle pulp and to a sample freed of co-enzyme showed the oxygen uptake of the latter to be twice that of the former.

Ascaris has only a very small catalase content, and if this enzyme is added to muscle pulp there is a rise in oxygen uptake, especially if methylene blue is added as well. This effect is more marked if co-enzyme-free pulp almost free from catalase is used. The possible formation of hydrogen peroxide was, therefore, investigated and proof was obtained that it is present in muscle pulp whose oxygen uptake had been raised by addition of methylene blue or by exposure to pure oxygen.

Worms exposed to pure oxygen die rapidly, and this may be due to accumulation of hydrogen peroxide whose presence under these conditions was shown.

The effect of potassium cyanide and sodium nitrate on the oxygen consumption of muscle pulp and of whole worms was studied. In neither case was there evidence that these substances harm the respiratory system. Potassium cyanide in high concentrations actually increases the oxygen uptake of the muscle pulp, probably because it combines with oxaloacetic acid. The death of worms injected with potassium cyanide and sodium nitrate was probably caused by secondary effects.

Laser points out that the oxygen consumption of *Ascaris* in air is of the same order of magnitude as of a resting man, and that of its muscle pulp is nearly the same as of mammalian muscle pulp. The oxygen consumption of *Ascaris* must be much smaller at the oxygen tensions encountered in the normal environment. The appreciable oxygen consumption at so low a tension as 5 % and the development of an oxygen debt after anaerobiosis support the view that the animal requires a certain amount of oxygen rather than that it is a facultative aerobe.

The small amount of glycogen present, the inability to break down oxaloacetic acid, the formation of hydrogen peroxide and the low catalase content all support Laser's general conclusion that the respiratory system present 'is particularly adapted to low rates of oxidation' and to the low oxygen tensions occurring in the normal environment.

Weinland (1901*b*) found in *A. lumbricoides* under anaerobic conditions, the following quantitative relations between the carbohydrate consumed and the excretory products formed:

0·7 g. glycogen + 0·1 g. dextrose
= 0·04 g. CO_2 + 0·3 g. $C_5H_{10}O_2$.

Considering the carbohydrate as sugar, he suggested the process of breakdown might be

$$\underset{720}{4C_6H_{12}O_6} = \underset{396}{9CO_2} + \underset{306}{3C_5H_{10}O_2} + \underset{18}{9H_2}.$$

Since he did not observe the formation of free hydrogen he suggested that it might, *in statu nascendi*, react with some unknown substance in the animal. He concluded that, in any case, the carbohydrate must be broken down by a fermentation process such as is found in bacteria and yeasts since *Ascaris*, in its natural surroundings, has not access to sufficient oxygen for the ordinary combustion, the sole end-products of which are carbon dioxide and water. Weinland also pointed out the extreme inefficiency of this fermentative breakdown, which, he estimated, could not produce more than 25 % of the energy resulting from combustion, and therefore is only possible for an animal to which an unlimited supply of food is available.

Weinland (1901*b*) also considered the possible equation

$$13C_6H_{12}O_6 = 12C_5H_{10}O_2 + 18CO_2 + 18H_2O,$$

suggested by Koenig. This, however, would not yield the amount of carbon dioxide found in his experiments, although, as Brand & Jahn (n.d.) point out, this might be derived from bicarbonate or protein. Brand & Jahn quote a chain of reactions suggested by Jost which could lead theoretically to Koenig's equation. These are of interest, as they point out, because the first stage in the carbohydrate breakdown is the production of lactic acid which is thus connected with the formation of valeric acid.

Toryu's (1936) equation

$$4C_6H_{12}O_6 = 4CO_2 + 4C_5H_{10}O_2 + H_2O,$$

is, as Brand & Jahn note, unacceptable as it does not balance.

The similarity of the excretory products of nematodes such as *Ascaris* under both aerobic and anaerobic conditions has led to the conclusion that

fermentative processes continue even when oxygen is present. Brand (1934) calculated the following balance sheet for *Ascaris lumbricoides*:

I. *Under anoxybiotic conditions:*

100 g. of fresh worm consume in 24 hr. 1·39 g. glycogen + 0·18 g. protein, of which the end-products are 0·71 g. CO_2 + 0·22 g. valeric acid + 0·02 g. lactic acid + 0·02 g. N in dissolved excretory material + 0·01 g. N in eggs.

II. *Under oxybiotic conditions:*

100 g. of fresh worm consume in 24 hr.

(*a*) By oxybiotic metabolism 0·37 g. glycogen (*c.* 0·21 g. O_2 taken up: R.Q. = 0·9) of which the end-products are 0·34 g. $CO_2 + x$.

(*b*) By anoxybiotic metabolism consume 0·86 g. glycogen + 0·17 g. protein of which the end-products are 0·48 g. CO_2 + 0·16 g. valeric acid + 0·01 g. lactic acid + 0·02 g. N in dissolved excretory material + 0·01 g. N in eggs.

In the oxybiotic metabolism the breakdown products of the carbohydrates were not identified. The relation found was

$$C_6H_{12}O_6 + 3O_2 = 3CO_2 + x.$$

Brand suggested that aldehydes might be among the unknown breakdown products designated by x in the balance sheet.

Little is known of the metabolism of parasitic nematodes other than *Ascaris lumbricoides* and *Parascaris equorum*, but it is interesting to note that Stannard *et al.* (1938) found that when mature larvae of *Trichinella spiralis* are released from their cysts fermentation also occurs side by side with respiration under aerobic conditions. In this case, however, the products of fermentation are carbon dioxide and other non-acid substances which have not been identified.

VII. THE SURVIVAL OF PARASITIC NEMATODES UNDER ARTIFICIAL CONDITIONS

(1) *Introduction*

It is probable that the greatest single obstacle confronting the investigator of the physiology of nematode parasites of animals is the difficulty of keeping the worms alive and healthy for long periods apart from their hosts. It is well known that nematodes are usually very resistant to unfavourable environmental conditions, and it is possible to keep some parasitic species alive and active in the laboratory for periods ranging from a maximum of about 1 month in the case of intestinal parasites of warm-blooded animals to several months in the case of some blood and tissue inhabiting forms. This characteristic hardiness has made possible many valuable observations, but the comparatively short life of the worms under experimental conditions and the changes which occur in their behaviour, metabolism and chemical composition are reminders that we can hardly regard most of the specimens which have been investigated experimentally as 'normal and healthy'. Results obtained from them should therefore be interpreted with caution.

Only two species of nematodes parasitic in animals have been reared *in vitro* throughout their life history, and these are both parasitic in insect hosts. Glaser (1931) was able to cultivate *Neoplectana glaseri* from the Japanese beetle, and Glaser, McCoy & Girth (1942) successfully cultivated an allied species, *N. chresima*. Brand & Simpson (1944) have kept the larvae of *Eustrongylides ignotus* alive *in vitro* for as long as 2½ years, and report the case of a single male individual which developed to the adult stage. The larvae of this species are tissue parasites in the fish, *Fundulus heteroclitus*, while the adults live in the black-crowned night heron, *Nycticorax nycticorax hoactli*.

The following are records of the longest periods of survival *in vitro* of various species: an unidentified nematode from the connective tissue of *Scomber scombrus* for 3 months (Dewitz, 1899); the microfilaria of *Setaria labiato-papillosa* for 52 days (Johns & Querens, 1914); the microfilaria of *Dipetalostoma perstans* in an ice-chest for 4–5 weeks (Fülleborn, 1912); the microfilaria of *Wuchereria bancrofti* in an ice-chest for 6–7 weeks (Fülleborn, 1912); larvae of *Trichinella spiralis* for 4 months (Levin, 1940). Among intestinal nematodes Magath (1919) kept *Camallanus americanus* alive for about 2 months, *Ascaris lumbricoides* has lived for as long as 26 days (Hall, 1917) and unsheathed infective larvae of *Haemonchus contortus* for 3 weeks (Glaser & Stoll, 1938*b*).

This list is by no means exhaustive and other examples will be mentioned later, but the examples quoted indicate that the species parasitic in cold-blooded animals seem to live longer in artificial media. Among the species parasitic in warm-blooded hosts the microfilariae are peculiarly resistant although they have survived for the longest periods in an ice-chest. Species parasitic in the alimentary tract of mammals and birds have been the most difficult of all to keep alive apart from their hosts. Perhaps they are adversely affected by the number of bacteria usually present in the media in which they have been kept.

In the following pages attention will be concentrated upon the chemical and physical conditions which influence the life of the parasites kept *in vitro*. The special problem of the factors controlling ecdysis and the exsheathing of infective larvae of the Strongyloidea will not be considered in detail.

Lapage (1937) has summarized the considerable literature on the subject. Nor will the cultivation of free-living larval stages be considered except in so far as it is important in relation to the study of parasitic phases.

(2) *The inorganic salts of the culture medium*

All workers who have studied parasitic nematodes under laboratory conditions have used some kind of a 'physiological salt solution', either as the only artificial medium which they have employed or as the basis of other media. For short-term physiological experiments 'physiological salt solution' alone has been often used.

Numerous investigators, from Bunge up to the present day, have used simple solutions of sodium chloride as their medium. Bunge (1883) found that the majority of specimens of *Toxocara mystax* lived for 7–10 days and some for 13–14 days in 1 % NaCl at 38° C. under aerobic conditions. A slight improvement was made by the addition of 0·1 % Na_2CO_3. Weinland (1901*b*) also used 1 % NaCl for his experiments with *Ascaris lumbricoides*, but only succeeded in keeping the worms alive for 3–5 days when oxygen was present. He found that the addition of sodium carbonate made no difference, but that saturation of the medium with carbon dioxide increased the period of survival to 7–9 days. Hall (1917) put nine specimens of *A. lumbricoides* into physiological sodium chloride solution and found that the last one, a male, died on the 15th day. Nine specimens in Kronecker's solution (physiol. NaCl + 0·06 g. NaOH/l.) lived longer, three females being alive after 19 days, two after 24 days and one survived 26 days. These survival times are the more remarkable since the medium was not changed, distilled water being added to replace loss of fluid evaporation. It is noteworthy, however, that the temperature was only 27·5–30° C. Enigk (1938) kept exsheathed third-stage larvae of *Graphidium strigosum* alive for 5 weeks in sodium chloride solution and fourth-stage larvae of the same species for 18–21 days.

The majority of workers who have made experimental investigations of the physiology of *Ascaris lumbricoides* and *Parascaris equorum* (e.g. Schimmelpfennig, 1903; Kemnitz, 1912; Schulte, 1917; Fischer, 1924; Harnisch, 1933, 1935; Hoffmann, 1934; Waechter, 1934; Krüger, 1936; Brand, 1937*a*; Oesterlin, 1938), have been content to use sodium chloride solution as a medium for the nematodes. This is somewhat surprising because the importance of balanced salt solution is generally recognized in physiological work.

Some workers, however, have used balanced solutions. Dewitz (1899) kept an unidentified nematode parasitic in the connective tissue of *Scomber scombrus* for 3 months in sea water, and Seurat (1919) found that *Proleptus obtusus* from *Scyllium catulus* survived for a maximum of 23 days in the same medium. For short-term experiments on *Ascaris lumbricoides*, Slater (1925) used Ringer, Adam (1932) used Tyrode's solution and Hirsch & Bretschneider (1937) used Tyrode as well as pure sodium chloride solution. Toryu (1934–6) used Ringer for the study of *Parascaris equorum*.

Davey (1938*b*) investigated the action of salt solutions upon adult *Ostertagia circumcincta*. He found that in 0·9 % NaCl the survival time never exceeded 24 hr. Addition of 0·024 % $CaCl_2$ increased the length of life to 3 days. If 0·042 % KCl was also present the solution was as good as Ringer-Locke, in which the nematodes lived 4–12 days. He noted, however, that 'even when the only calcium present in the medium was derived from impurities—an amount not exceeding 0·0005 %—the addition of 0·042 % KCl made the solution apparently as good as the full Ringer-Locke solution'. The addition of Na_2HPO_4, KH_2PO_4 or $MgCl_2$ had no effect. His basic saline medium was, therefore: NaCl 0·9 %, KCl 0·042 %, $CaCl_2$ 0·024 %.

Fenwick (1939) worked with sterile larvae of *Ascaris lumbricoides* obtained by causing the eggs to hatch by treating them with 1 : 15 Milton solution for 12 hr. at 38° C. In an isotonic (0·832 %) sodium chloride solution the larvae survived 2–2½ days. In a sodium chloride solution with 0·02 % KCl the survival time was 3¾–4 days. Addition of 0·020 % $CaCl_2$ to the NaCl + KCl caused a further increase in survival time to 4¾ days. The larvae were not indifferent to the presence of magnesium as were the adult *Ostertagia* used by Davey, since the addition of 0·01 % $MgCl_2$ caused the *Ascaris* larvae to live for 5½ days. Fenwick tested the effects of varying the proportions of the four salts and arrived at the following saline medium: NaCl 0·80 %, KCl 0·02 %, $CaCl_2$ 0·02 %, $MgCl_2$ 0·01 %.

For the maintenance of *Ascaris lumbricoides* in the laboratory Baldwin (1943) made up the following stock solution: NaCl 80 g./l., KCl 2 g./l., $CaCl_2$ 2 g./l., $MgSO_4.7H_2O$ 1 g./l. To 1 vol. of this solution was added $\frac{1}{10}$ vol. of 0·05 M phosphate buffer *p*H 6·4, and it was then made up to 10 vol. with distilled water. In this medium the nematodes would live for 10–11 days, and Baldwin adds that 'Although the animals are undoubtedly in a state of extreme physiological decrepitude towards the end of this period, there is no reason to doubt that for the first 48 hr. at any rate they are for the most part in a physiological condition closely approximating to normal. . . .' This medium was also employed by Rogers (1945). Lapage (unpublished work) used it in the course of studies of the effects of anthelminthic substances upon *A. lumbricoides* and other species.

Hobson, Stephenson & Beadle (unpublished work) have used 30 % sea water as a medium for the study of adult *A. lumbricoides*. This fluid, the composition

of which is given in Table 19, proved to be as successful as any of the media which had been used by earlier workers. Individual specimens of *A. lumbricoides* survived in it for as long as 28 days. It is interesting to note that this medium, like the normal environment of *A. lumbricoides* and like Fenwick's saline medium, contains magnesium. In later experiments which are still in progress Hobson & Hobson have been able to increase still further the survival time of apparently healthy *A. lumbricoides* by using an artificial 30 % sea water with the addition of nutrient material.

Baldwin & Moyle (1947) have recently used a saline medium for keeping *A. lumbricoides* alive *in vitro*, which is based upon Eden's unpublished analyses of the intestinal contents of the pig (see Table 1 of this review). This medium differs chiefly from the one which Baldwin (1943) used previously in its much greater content of potassium. It is buffered with phosphate to *p*H 6·7. Baldwin & Moyle state that the worms remain more active in this new medium but do not live longer. Individuals remained alive for 4–12 days.

Table 19

Medium	Na (mmol.)	K (mmol.)	Ca (mmol.)	Mg (mmol.)	Cl (mmol.)	SO_4 (mmol.)
Davey's saline	154	6	3	Nil	166	Nil
Fenwick's saline	137	3	2	2	148	Nil
Baldwin's saline	136	3	2	0·4	141	0·4
Baldwin & Moyle's saline	130	24	6	5	170	Nil
30 % sea water	141	3	3	16	164	8·5

The relative composition of the five media is shown in Table 19.

The media are, in general, similar. The most striking differences are the high magnesium concentration of the diluted sea water medium, the complete absence of this substance from Davey's mixture and the relatively large amount of potassium in Baldwin & Moyle's saline.

Stoll (1940*a*) used Tyrode's solution for study of the larvae of *Haemonchus contortus*, and notes that normal saline is toxic. The same author (1940*b*) compared the effects of Tyrode's solution, Ringer's solution and normal saline on the same species and confirmed his observations that the larvae live longer in a balanced salt solution than in sodium chloride alone. The percentage of third-stage larvae which lived for 5 weeks or more was greater in Tyrode's than in Ringer's solution, although there was little difference during the first 3 weeks of the experiments. Similar results were obtained if glucose was omitted from the Tyrode's solution. The larvae survived longer if the salt solutions were diluted to about one-quarter to three-quarters of their normal concentration.

It is probable that, in the past, too little attention has been paid to the electrolyte composition of the medium in which parasitic helminths are kept. It has already been shown that the composition of the body fluid of *Ascaris lumbricoides* is affected by that of the environment. Such alterations in the intestinal environment cannot be supposed to have anything but an unfavourable effect upon the nematode, and are probably responsible to some extent for its short time of survival in saline media.

(3) *Oxygen*

The relation of oxygen to parasitic nematodes has been discussed in a previous section. Although the evidence bearing on the problem is rather scanty there is little to show that parasitic worms are benefited *in vitro* by anaerobic conditions. Toryu (1935), working with *Parascaris equorum*, is the only investigator who has found that the survival time is shortened by the presence of oxygen. It has been established by the work of Adam (1932), Brand (1934), Krüger (1936) and others that *Ascaris lumbricoides* uses oxygen when it is available. These observations, together with the earlier ones of Slater (1925), indicate that this species should be regarded at least as a facultative aerobe. Laser (1944) even suggests that a small amount of oxygen is required by this species. The investigations of Wells (1931) on *Ancylostoma caninum*, of Davey (1938*a*) on various species of nematodes from the alimentary canal of the sheep, and of Stannard *et al.* (1938) on the larvae of *Trichinella spiralis*, indicate clearly that oxygen plays an even more important role in the life of these smaller species. While the evidence favours the view that, for the majority of the small number of species which have been examined, the presence of air under experimental conditions is better than its complete absence, it does not follow that this necessarily gives the optimum oxygen tension. Intestinal parasites can never encounter, in their natural surroundings, oxygen tensions approaching that of air, and it may be that they would be more healthy *in vitro* if the oxygen concentration were adjusted accordingly. No experiments have, however, been made to determine the length of survival of nematodes kept at various oxygen tensions below that of air.

(4) *Carbon dioxide*

Weinland (1901*b*) observed that saturation of the medium (1 % NaCl) with carbon dioxide caused an

appreciable increase in the survival time of *Ascaris lumbricoides* in this medium. He explained this by reference to the high carbon dioxide content of the normal environment. Following Weinland, Schulte (1917) and Waechter (1934) used saline solutions saturated with carbon dioxide for their experiments but made no observations upon its effect on the length of life of the worms. Toryu (1935) found that carbon dioxide was definitely beneficial to *Parascaris equorum*; the worms lived two to three times as long in a medium (Ringer) saturated with carbon dioxide as in one saturated with oxygen. More observations are needed on this matter, but Brand & Jahn (n.d.) have pointed out that carbon dioxide may be concerned with the regulation of the intracellular *p*H of the worms and that, if this is so, it may be important that there should be a high carbon dioxide tension in artificial media.

(5) *Hydrogen-ion concentration*

A few workers have studied the effect of variations in the hydrogen-ion concentration of the medium. Intestinal parasites must be exposed to considerable fluctuations in the *p*H of their natural surroundings so that it is to be expected that they will tolerate a fairly wide range of *p*H. Lapage (1935) found that exsheathed parasitic larvae of *Haemonchus contortus*, *Ostertagia circumcincta* and *Trichostrongylus* spp. could withstand *p*H values between 3·6 and 9·6. Davey (1938*b*) investigated eight species of nematodes from the alimentary tract of the sheep and found that the range of *p*H tolerated was in every case wide, although there were characteristic differences between the various species. Thus, for *Ostertagia circumcincta*, the range was *p*H 3·2–9·0, while for *Cooperia oncophora* it was *p*H 4·0–8·5. Enigk (1938) found that the exsheathed third-stage larvae of *Graphidium strigosum* lived longest in a medium of *p*H 3·0–4·0; at *p*H 8·0 or above they died in 24 hr.; at *p*H 1·0 they were very active but died in 2 days. For the fourth-stage larvae of this species the optimum *p*H was 4·0–5·5, and between these limits the *p*H had little effect upon the larvae. Whitlock & Leasure (1938) found that adult *Strongylus vulgaris* lived longer in Locke's solution at *p*H 6·8–7·5 than at *p*H 4·0–5·4. Later Whitlock *et al.* (1939), using the same material, examined the question in more detail. Using 0·85 % NaCl, Locke's and Ringer's solutions as media and buffering with MacIlvaine's, with Britton and Robinson's universal and with phosphate buffers they found a distinct relation between the external hydrogen-ion concentration and the length of life of the nematodes. They found the optimum range to be *p*H 6·5–8·0 which agrees with that of the normal environment of this species. An irreversible toxic effect was caused by exposure of the animals to *p*H 4·0–5·5 for 4 hr. or more. No observations on the *p*H tolerance of adult *Ascaris lumbricoides* have been published, but Fenwick (1939) found that the larvae in a balanced saline medium could withstand *p*H 5·0–9·0.

Brand & Simpson (1944) found that larvae of *Eustrongylides ignotus*, which are parasites in the tissues of the fish *Fundulus heteroclitus*, survived longest *in vitro* when the medium was about neutral but could tolerate a wide range of hydrogen-ion concentration. They also confirmed with this species the observations of Whitlock *et al.* (1939) that *Strongylus vulgaris* can alter the *p*H of the medium in the direction of that in which it lives normally. When the larvae of *Eustrongylides ignotus* were kept in extremely acid media there was a rise in *p*H while there was a fall when the media were alkaline.

It seems, therefore, that the hydrogen-ion concentration of the medium must be taken into consideration when attempting to cultivate parasitic nematodes. In the alimentary canal of the host rapid changes of *p*H take place and the nematode must be able to withstand these and so must possess a wide range of tolerance for relatively short periods of exposure to these variations. It does not follow, however, that this same range can be tolerated under conditions of artificial cultivation which are designed to maintain a fairly constant hydrogen-ion concentration. In these circumstances the range of tolerance may be more limited than the available experimental results indicate.

(6) *Oxidation-reduction potential*

Jahn (1933) pointed out that it has been possible to cultivate many intestinal Protozoa only when bacteria are present. He suggested that, in some cases, the function of the bacteria might be to cause a necessary decrease in the oxidation-reduction potential of the medium which may be too high for the parasite. When, therefore, it is desirable to eliminate bacteria, media of lower potential might be tried. Kollath & Erhardt (1936), quoted by Hoeppli *et al.* (1938), emphasize the importance of the oxidation-reduction potential in the cultivation of the trematode *Opisthorchis felineus*. They found that this species lived for 21 days in pure Ringer's solution but for 40 days if 1 : 50,000 potassium-indigotinsulphonate was present. No observations have been made on the possible importance of this factor in the cultivation of parasitic nematodes.

(7) *Osmotic pressure*

It has been somewhat generally assumed that the osmotic pressure of an internal parasite must be similar to that of its environment. While this may be, and probably is, true in the case of nematodes living in the blood and other tissues of the host, it is not correct in the case of the one intestinal species which has been adequately investigated. Schopfer and others (see an earlier section of this review) showed

clearly that *Ascaris lumbricoides* is distinctly hypotonic to the intestinal fluid of the pig in which it lives. When this species is transferred from its normal environment to a saline solution of the concentration which produces minimal change in body weight, there is a reversal of the osmotic gradient across the body wall. The body fluid becomes slightly hypertonic to the external medium.

Little work has been done on the effect of variations of external osmotic pressure on the length of time for which a worm can survive under artificial conditions. Bunge (1883) found that for *Toxocara mystax* 1 % NaCl was the optimum concentration. The survival time of this species in 0·5 or 1·5 % NaCl was distinctly shorter, and in 3 % NaCl it was only 1 day. Davey's (1938*b*) work on *Ostertagia circumcincta* showed that this species was apparently unharmed by balanced salt solutions (see above) whose total concentrations were equivalent to not more than 1·3 % and not less than 0·4 % NaCl. Similarly, Fenwick (1939) found that the larvae of *Ascaris lumbricoides* were not very sensitive to osmotic pressure and could withstand concentrations of balanced salt solution lying between the sodium chloride equivalents of 0·7 and 1·1 %.

The small amount of evidence available suggests that parasitic nematodes are not very sensitive to changes in osmotic pressure, and that those species which inhabit the alimentary canal must be subjected to considerable variations of this factor. Yet the work previously cited of Schopfer (1932 and earlier papers), Panikkar & Sproston (1941) and Hobson, Stephenson & Beadle (unpublished) indicates that these species are poikilosmotic. Temporary variations of osmotic pressure about a mean value which occur under natural conditions may well have very different effects upon the parasites than those exerted under experimental conditions by a constant deviation from this mean. However, the reversal of the osmotic gradient which is caused by the removal of *A. lumbricoides* from its normal environment to a saline medium (see an earlier section of this review) may reasonably be supposed to have far-reaching effects which do not favour the long survival of the animal. If this reversal can be prevented by the addition to the medium of non-electrolytes and colloidal material the period of survival of the worms *in vitro* may be lengthened. It may be noted, however, that Davey (1938*b*) found that the addition of isotonic glucose solution to the saline medium was not advantageous to *Ostertagia circumcincta*.

(8) *Temperature*

The relation of temperature to nematode parasites of warm-blooded animals is important and peculiar. They share with many other internal parasites of mammals and birds the unusual feature of being exposed to very different temperatures at different stages of their life history. The evolution of their adaptation to the high temperature of the warm-blooded host has been briefly discussed by Jammes & Martin (1910).

Bunge (1883) noted that *Toxocara mystax* at 15–20° C. became stiff and motionless. At first they could be made active by warming but they died in 2–3 days. Weinland (1901*b*) made similar observations on *Ascaris lumbricoides*. He also pointed out that the worms are sensitive to fluctuations of temperature, and that if the temperature of the medium was raised above 40° C. they were killed within a period varying from a few hours to 1 day. Since then it has been generally recognized that, although intestinal nematodes should be cultivated at the temperature of the host, about 38° C., any increase above this is harmful. Intestinal nematodes are evidently living very close to the upper limit of their temperature range, and attempts have been made to take advantage of this to eliminate parasites from the alimentary canal of the host. De Rivas (1926*a*) found that various nematodes and cestodes of the dog were killed in 12 hr. at 40° C., in 10 min. at 45° C. and in 5 min. at 47° C. He described (1926*b*, 1932) successful treatment of infections with intestinal Protozoa, cestodes and nematodes in the dog and man by means of intestinal lavage at temperatures of 42–47° C. Hall & Shillinger (1926) found that this method of treatment was not so successful as claimed by de Rivas. Otto & Abrams (1939) experimented with diathermic treatment on cats infected with *Trichinella spiralis* and found that the hosts were killed before the larvae.

Parasites of the blood and tissue of warm-blooded animals seem to be more resistant to low temperature than are those which inhabit the alimentary tract, although it is doubtful whether such a generalization is justified in view of the comparatively small number of species which have been investigated. It is well known that encysted larvae of *Trichinella spiralis* are particularly resistant. Schmidt, Ponomara & Savelier (1915) found that they were unaffected at 0° C.; at −6° C. emergence from the cyst when treated with gastric juice was slightly delayed; at −9° C. some, and at −15° C. all, were killed. Ransom (1916), in an elaborate series of experiments on the effects of refrigeration, showed that after 23 days at 15° F. many larvae survive, but there is some loss of infectivity; at 5° F. and lower, few survive and infections are rare. Augustine (1932) investigated the effect of quick freezing of heavily infected pork and found that a few larvae remained infective after exposure to −30·9° C.; at −33·7° C. about 12 % of the larvae appeared normal and reacted to warming but none was infective. Levin (1940) found that larvae kept in Tyrode's solution for 4 months at 5° C. gave normal infections; the experiment was not continued after this period.

Microfilaria larvae are also remarkable for their power of surviving *in vitro* for long periods at temperatures much below those to which they are accustomed in their normal environment. Fülleborn (1912) recorded that when the microfilariae of *Dipetalonema perstans* and *Wuchereria bancrofti* were kept in an ice-chest they survived for 4–5 weeks and 6–7 weeks respectively, and Wellmann & Johns (1912) obtained the best results when they cultivated the larvae of *Dirofilaria immitis* at 12° C.

If we exclude the species of *Neoplectana*, the longest period for which any parasitic nematode has been kept alive *in vitro* is 2½ years. Brand & Simpson (1944) kept the larvae of *Eustrongylides ignotus* alive for this period at 20° C. They survived at this temperature much longer than at 37° C. and evidence is presented that the metabolism differs at the two temperatures (Brand, 1943; Brand & Simpson, 1944).

There is little evidence to show whether this capacity for surviving longer *in vitro* at subnormal temperatures is also characteristic of intestinal parasites, but the observations of Bunge (1883) and Weinland (1901*b*), cited above, suggest that it is not.

(9) *Light*

There is little evidence about the effect of light upon parasitic nematodes. Weinland (1901*b*) was uncertain whether *Ascaris lumbricoides* showed any sensitivity to light, but Toryu (1936*b*) recorded some remarkable observations on *Parascaris equorum*. He found that if oxygen was present the survival time and respiratory exchange in the light and in the dark were the same. The survival time was also unaffected by light if the worms were in a medium which contained no oxygen but was saturated with carbon dioxide. On the other hand, worms placed in a medium which was not saturated with carbon dioxide and was devoid of oxygen survived longer and produced more carbon dioxide in the dark than in the light.

(10) *The nutrition of parasitic nematodes in artificial media*

If parasitic nematodes are to be reared throughout their life history or even if the adult forms are to be maintained apart from their hosts for long periods in the laboratory, it is obvious that they must be supplied with suitable nourishment. Although some species can be kept for a time without food and have been extensively used in this condition for experimental purposes, they cannot live indefinitely upon their own reserves.

There is a considerable amount of evidence that non-parasitic nematodes feed largely upon bacteria, and this habit is probably shared by the free-living larvae of many parasitic species. McCoy (1929*a*, *b*) found that the free-living larvae of *Ancylostoma caninum* could be reared from hatching to the infective stage upon pure cultures of living bacteria belonging to species which are normally present in the faeces of their hosts. If the bacteria were killed the larvae would not develop. McCoy found that all species of bacteria were not suitable, since development of the larvae was slow or did not occur in cultures of *Bacillus cereus*, *B. megatherium*, *B. pyocyaneus* or *Sarcina lactea*. Lapage (1933) also found that the free-living larvae of several species of Strongyloidea developed normally on cultures of a strain of *Bacillus coli* which he had originally obtained from the gut of the parasitic larvae of one of these species. Glaser & Stoll (1938*a*), modifying the method introduced by Lapage (1933*a*), reared larvae of *Haemonchus contortus* to the infective stage under sterile conditions on a medium containing killed and ground yeast, liver extract and pieces of rabbit kidney. The larvae were rather small but normally infective.

There is little evidence that the parasitic stages of even intestinal nematodes use bacteria for food, although Lapage (1937) quotes the statement by Hilgermann & Weissenberg (1918) that *Nematoxys ornatus*, a species parasitic in the large intestine of the frog, develops 'well on agar plates inoculated with colon-bacteria from their hosts'.

Glaser (1931) was the first worker who succeeded in rearing a parasitic nematode through the whole of its life history. He successfully cultivated *Neoplectana glaseri*, a parasite of the Japanese beetle, *Popillia japonica*, on standard meat infusion agar plates containing 1% dextrose at *p*H 7·4. A suspension of an actively growing yeast was added with the gravid female worms used to start the culture. In a later paper Glaser (1940*a*) described a method of cultivating the same species under sterile conditions on neutral veal infusion agar slants or in 2% agar prepared with 0·5% NaCl. Whichever medium was used a piece of animal tissue removed under sterile conditions was added to it as a nutrient. Rabbit kidney was found to be the most satisfactory, but 18–20-day-old mouse embryo, beef kidney and rabbit ovary all supported growth. These cultures would continue for 3 months without transplantation, but it was found better to transplant every 3 weeks. Prolonged culture under these conditions resulted eventually in dying down owing to the appearance of sterile females (Glaser, 1940*b*). This could be prevented by passing the worms through the host. Revival of a waning culture could be brought about by the addition of living yeast, or of a powder formed from dried Japanese beetle larvae or beef ovary. The best results were obtained with a combination of living yeast and dried beef ovary. Nine or ten transplants had to be made before the culture was restored to full vitality, and Glaser concluded that, when the culture is started, the

nematodes contain a growth factor which becomes depleted during successive generations apart from the host. This growth factor can be restored, either by passing the worms through the host or by treating the standard medium with the materials named. In the latter case the treatment has to be continued through many generations.

Glaser, McCoy & Girth (1942) have also succeeded in cultivating *Neoplectana chresima*, which is parasitic in various insects, by similar methods. A sterile culture in rabbit kidney was maintained for 2 years (28th transplant). This species would also live on an autoclaved medium of ground beef liver or kidney, water, sodium chloride and agar. Neither species of *Neoplectana* would grow on beef muscle.

Attempts to cultivate the parasitic stages or even to maintain the adults of nematodes of vertebrates have met with an almost uniform lack of success. Dewitz (1899) added cod-liver oil to the sea water in which he kept an unidentified nematode parasitic in the connective tissue of *Scomber scombrus* and thought that it was used during the 3 months during which the worm lived. He did not, however, determine how long the worms would live in pure sea water.

Wellman & Johns (1912) and Johns & Querens (1914) reported growth of microfilaria larvae of *Dirofilaria immitis* and *Setaria labiato-papillosa* kept in defibrinated blood and in blood serum with dextrose. Their results, however, have not been confirmed by other workers on the same species. Nagano (1923) could find no evidence of the growth of *Dirofilaria immitis* larvae in dextrose-blood medium at either 8–13° C. or 37° C.

Many attempts have been made to cultivate the microfilaria of *Wuchereria bancrofti*. Fülleborn (1912) preserved them for 6–7 weeks in coagulated human blood in an ice-chest. Takeshita & Okuda (1925, quoted by Hoeppli *et al.* 1938) kept them for 23 days in 'cattle haemoglobinized saline' at 22–27° C. Coutelen (1929) found that they lived for 32 days in fresh human serum. Menon & Ramamurti (1940) tried a number of organic media but obtained the maximum survival of 50 days in normal saline solution at 10° C. They observed no growth in any of the media which they used.

Fenwick (1939) found that glucose in concentrations of 1 % or less had no effect on the life of *Ascaris lumbricoides* larvae in saline media. In higher concentrations it was toxic.

Levin (1940) found that the larvae of *Trichinella spiralis* would live for 11 days in Tyrode's solution at 38° C. At 5° C. they lived for 4 months or longer, and Levin thought that solutions containing glucose were better for them over long periods. Stannard *et al.* (1938), however, could obtain no evidence that the larvae of this species used glucose in the medium. Their experiments were carried out at 37·5° C. The work of McCoy on the same species is important. He (1933–4) found that sterile larvae introduced into the amniotic cavity of chick and rat embryos could develop to maturity. Only 1–2 % of the larvae developed successfully, and the adults produced were less than the normal size. He also (1936) obtained similar development of larvae in the uterus of pregnant rats. He pointed out that these experiments show that the presence of bacteria, digestive enzymes or partly digested food is not necessary for the growth of the larvae. They would not, however, develop in sterile amniotic fluid *in vitro* (1933–4).

Attempts to cultivate intestinal nematodes or their larvae in 'nutrient' media have not been successful. The experiments of Weinland & Ritter (1902), Hoffmann (1934) and Hobson, Stephenson & Hobson (unpublished) suggest that *Ascaris lumbricoides* can absorb and probably consume certain sugars present in the external medium. Davey (1938*b*) found that the addition of glucose did not increase the period of survival of *Ostertagia circumcincta* in a saline medium.

Magath (1919) succeeded in keeping *Camallanus americanus*, parasitic in the turtle, alive for 2 months. He employed as media, first, Witte's peptone and gelatine and, secondly, turtle blood-agar.

Lapage (1933*a*, 1935) isolated the sterilized infective larvae of *Haemonchus contortus*, *Ostertagia circumcincta* and *Trichostrongylus* spp. in hanging drops of more than 200 different sterile media. None of these larvae grew, but most of them developed until they were ready for the third ecdysis. Only ten out of more than 1500 larvae performed their third ecdysis, but all the ten fourth-stage larvae thus liberated died. The larvae usually lived 18–30 days in the hanging drops before vacuolation and emptying of the intestinal cells appeared. These changes in the intestinal cells usually indicated the approach of death. None of the larvae exhibited any particular reaction to the presence of blood or its derivatives, or of the mucosa of the host's stomach and duodenum in the media.

Ackert, Todd & Tanner (1938) tried to cultivate the larvae of *Ascaridia galli* (=*lineata*) taken from the duodenum of chickens. They had a solid dextrose-cornmeal-agar plate covered with sterile salt solution. The larvae survived for nearly 2 weeks. Addition of peptone was unfavourable. In a fluid medium composed of starch, maltose and peptone in distilled water the larvae died in 2 days and did not grow. Growth was, however, observed in a starch-dextrose-distilled water mixture and in the isotonic salt-dextrose medium used by Stunkard (1932) for the cultivation of the cestode *Crepidolothium lönnbergi*. Controls kept in isotonic salt solution showed a decrease in size. The larvae also grew if they were introduced into incubating hen eggs. They would not grow in the body cavity of chicks (Ackert, 1938).

Attempting to cultivate the third exsheathed larvae of *Graphidium strigosum*, Enigk (1938) examined the effect of the addition of various organic materials to physiological sodium chloride solution. Glucose, sucrose, rice and potato starch, albumen, fibrin and peptone were tried, but none of these substances enabled the larvae to live longer than they did in plain salt solution. The addition of pepsin, rennet, pancreatin, fresh blood, serum or washed red blood corpuscles was also ineffective. Most of these substances, and others also, were tried by Lapage (1935), whose results have been outlined above. Enigk found, however, that the length of life of the fourth-stage larvae in salt solution was increased if glucose was present, and there was a further improvement when 0·5–2 % albumen was added. Rabbit's serum had a similar effect. The final moult never occurred.

Glaser & Stoll (1938*b*), having, as mentioned above, succeeded in raising the larvae of *Haemonchus contortus* from the egg to the infective stage, attempted to continue the development further, *in vitro*. Exsheathment and sterilization of the infective larvae was accomplished by 1 : 20 Labarraque's solution. The sterilized larvae were then incubated in 0·5 % agar in Ringer's solution containing sheep-liver extract, heat-killed ground yeast, sheep blood and sheep kidney, the last being in the form of extract or as small pieces. At the end of 3 weeks the larvae had increased in size to about five times their initial length and had moulted to give the fourth (second parasitic) stage larvae.

Stoll (1940*b*) found that the ecdysis of the third-stage larvae of *Haemonchus contortus* would take place in Tyrode's solution, whether glucose was present or not, or in Ringer's solution. More favourable results were obtained if these solutions were diluted to one-quarter to three-quarters of the normal concentration. Veglia (1916, quoted by Stoll) found that, in the sheep, 70 % of the larvae of *Haemonchus contortus* had reached the fourth-stage 48 hr. after infection. In Stoll's experiments few of the larvae in the balanced salt solutions moulted before the end of the second week. The addition of aqueous liver extract to the Tyrode's solution accelerated ecdysis, but none of the fourth-stage larvae lived. Stoll also obtained some evidence suggesting that ecdysis occurs most readily in low oxygen concentrations.

Brand & Simpson (1944) found that the period of survival *in vitro* of larvae of *Eustrongylides ignotus* is increased by the addition to the basic medium (1·0 % Bacto-Peptone in 0·5 % NaCl) of 0·5 % glucose, xylose and, possibly, inulin. Cellobiose, mannose, laevulose and maltose were not effective. They also showed that the consumption of reserve glycogen was greater when no sugar was present in the medium.

There is a possibility that the failure to cultivate parasitic nematodes *in vitro* is due to the absence of substances which are essential for growth. Ackert & Beach (1931) and Beach & Ackert (1932) found that feeding chicks, infected with *Ascaridia galli*, with vitamin B in the form of brewer's or baker's yeast does not cause additional growth of the parasites provided the host is provided with an adequate diet. Levin (1940) found that the addition of vitamin B complex did not help in the cultivation of the larvae of *Trichinella spiralis*. The histological evidence given by Hill & Smyth (1944) and Smyth *et al.* (1945) that *Toxocara canis* (= *Belascaris marginata*) accumulates vitamin C in the intestinal cells suggests that the presence of ascorbic acid may be necessary for keeping the worms alive for long periods *in vitro*. The work of Glaser (1940) on *Neoplectana glaseri*, however, provides the strongest evidence that the presence of some special 'growth factor' is required for their continued life and reproduction.

(11) *The effect of bile salts and of mucus*

While the problem of keeping parasitic nematodes alive apart from their host is essentially one of imitating the natural conditions as closely as possible, it must be remembered that all substances present in the normal environment are not necessarily favourable. Davey (1938*b*) found that the addition of bile salts to culture media was not advantageous, and pointed out that the toxicity of these substances varies with the species of nematode and is correlated with the region of the intestine inhabited by each species.

In a series of papers Ackert and his collaborators (see Ackert, Edgar & Frick, 1939; Frick & Ackert, 1941; Ackert & Edgar, 1940; Ackert & Frick, 1940; Eisenbrandt & Ackert, 1941) have discussed the age resistance of fowls to *Ascaridia galli* (= *A. lineata*), and find that it is correlated with the number of goblet cells in the intestine. The addition of autoclaved duodenal mucus from the fowl retards the growth of young worms in isotonic salt-dextrose medium. The amount of this growth-retarding agent is correlated with the age of the fowl. It is thermostable and is not removed from mucus by prolonged washing. Washings passed through a Berkefeld filter also contained the inhibitor. Eisenbrandt & Ackert (1941) also found that duodenal mucus from the dog or pig, added to nutrient salt solution caused rapid death of the nematodes. They suggested that this might be a factor in host specificity.

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A STUDY OF THE EFFECT OF LIGHT, TEMPERATURE AND SALINITY ON THE EMERGENCE OF *CERCARIA PURPURAE* LEBOUR FROM *NUCELLA LAPILLUS* (L.)

By GWENDOLEN REES, D.Sc., *Department of Zoology, University College of Wales, Aberystwyth*

(With 13 Figures in the Text)

Up to the present very few authors have directed their attention to factors influencing the emergence of larval trematodes from their snail hosts. Cort (1922) has made a study of the escape of cercariae from their hosts and the writer (1931) has examined the effect of light and temperature on the emergence of three species of larval trematodes from three species of fresh-water molluscs. More recently, Giovannola (1936) has investigated the effect of a reversal of light and darkness on the periodicity of emission of cercariae from three species of host snails. All these studies have been made on fresh-water molluscs. In the case of molluscs living between tide-marks on the shore, in addition to variation in temperature there exists another variable factor, namely, the salinity of the water. Many rock-pools situated in the intertidal zone are small and will therefore be subject to considerable variations in temperature and salinity at different times of the year and even for short periods of up to 12 hr. during individual days. In the summer months the temperature of the water in these pools may rise to a high degree and the high temperature would be maintained for a longer period near high-water mark than near low-water mark, where the period of exposure is less. Similarly, the decrease in temperature would be very marked during the cold winter months. As the water in the exposed pools evaporates the salinity increases and high up on the shore, between successive tides on hot summer days, it may become twice that of normal sea water. The pools are also exposed to rain and in the same period the salinity of the water may decrease considerably if the precipitation is heavy. The light factor would be the same for molluscs living in fresh and in salt water, but the reaction of various species of larval trematodes to the presence or absence of light varies. Some emerge from their hosts in larger numbers during the day-time, others during the night and some indiscriminately during periods of light and darkness.

The experiments described in the following pages have been made with the object of determining the effect of a variation in the periods of illumination and of temperature and salinity on the numbers of cercariae emerging from an infected species of gastropod mollusc living between tide-marks on the shore. The species chosen was *Nucella lapillus* (L.) parasitized by *Cercaria purpurae* Lebour, the larval stage of *Parorchis acanthus* Nicoll from the herring gull, *Larus argentatus* Pont. *Nucella lapillus* is very abundant on the shore at Aberystwyth and is fairly evenly distributed on the rocks and in the rock-pools in the intertidal zone. Older specimens are, as a rule, found near low-water mark, where the number parasitized is a little higher than it is farther up the shore. This is due to the fact that when the tide is out, gulls alight more frequently on the rocks near low-water mark, so that there the chances of the snail becoming infected are greater.

Specimens of *N. lapillus* were collected at low water, in large numbers, over a wide area of rocks and rock pools. They were brought into the laboratory and isolated singly in small glass dishes containing sea water, to ascertain whether or not they were infected. The collections were made in the morning so that the snails could be left undisturbed in their individual dishes for a period of several hours during the day. If the snails were infected cercariae began to appear in the water almost immediately, although on very cold days there was a delay of from 2 to 3 hr. Among the infected snails the degree of infection varied somewhat and so, for each experiment, specimens were chosen which were, as far as it was possible to judge, uniformly parasitized. All infected snails intended for experiments were kept in an aquarium in the laboratory for a period of 24 hr. before each experiment was begun, in order to acclimatize them to laboratory conditions. For the purpose of the experiments the snails were isolated singly in glass dishes of 100 ml. capacity. The sea water was changed at intervals of 12 hr., at 9 a.m. and at

9 p.m. Fresh sea water was obtained from the bay each day and the change was made by transferring the snails from the dishes in which they were contained to similar dishes, placed some time previously beside them, so that the temperature in both was the same. A small piece of *Fucus* was placed in each dish as food for the snails, as starvation over a period of days might have affected the numbers of cercariae developing and consequently emerging. The dishes were covered with coarse-meshed muslin stretched over wire frames, to prevent the snails from energing from the water, which reached almost to the top. Counts of cercariae were made at the end of each 12 hr. period, after the snails had been removed to their new dishes. *Cercaria purpurae* is large and can be seen quite easily with the naked eye when the glass dish is placed on a black background and suitably illuminated. The movements of the cercariae are slow and they were counted by being removed singly with a fine pipette. A few cercariae encysted on the bottom and sloping sides of the glass dishes and these could easily be counted. All dishes were washed thoroughly to remove any cysts before they were used a second time.

LIGHT

Experiment I. An experiment was first performed to ascertain whether *Cercaria purpurae* emerged from the host snail during the daytime or during the night, and whether there was any regular periodicity in its emergence. Four infected snails were therefore observed over a period of eight consecutive days, being exposed to alternating periods of 12 hr. darkness from 9 p.m. to 9 a.m., and 12 hr. light from 9 a.m. to 9 p.m. For comparative purposes this condition will be referred to as normal during the remainder of the experiments. Counts of cercariae were made at the end of each 12 hr. period, i.e. at 9 a.m. and at 9 p.m. During the course of the experiment the temperature ranged from 15 to 18° C., the rise occurring during the day. The salinity of the water may be described as normal, as it was obtained daily from the bay.

The results of this experiment are shown in Table 1, Exp. I, and Fig. 1. The figures in the table are the averages for the four snails. It can be seen that there is a very definite periodicity in the emergence of *C. purpurae* from *Nucella lapillus*. Large numbers emerge when the host snail is exposed to light and very few during the hours of darkness. The average number emerging during the day over the whole period of the experiment was 1381 and during darkness 27, and the difference is therefore considerable.

When the snails were transferred from dark to light at 9 a.m. cercariae did not appear in the water immediately. There was a delay of from 1 to 2 hr., after which they appeared, at first in small numbers, and then in increasing numbers until about midday, when they emerged in a fairly steady stream for the rest of the period of exposure to light.

Table 1. *The effect of light on the emergence of* Cercaria purpurae *from* Nucella lapillus

Duration of experiments 8 days. Figures are averages for four snails in each case. Counts at the end of each 12 hr. period.

Exp. I. Exposure to alternating periods of 12 hr. darkness, D (9 p.m.–9 a.m.), and 12 hr. light, L (9 a.m.–9 p.m.).

Exp. II. Exposure for 3 of the 8 days to continuous darkness (*).

Exp. III. Exposure for 4 of the 8 days to continuous light (†).

Exp. IV. Exposure for 4 of the 8 days to reversed periods of light and darkness (‡).

Period of observation		Numbers of cercariae			
		Exp. I	Exp. II	Exp. III	Exp. IV
Day 1	D	10	5	5	9
	L	1357	2020	1047	1025
Day 2	D	22	17	10	12
	L	1675	2	1086	2
Day 3	D	18	2	1102	473
	L	1234	2 *	53	9
Day 4	D	20	2	48	948 ‡
	L	1696	1	100	7
Day 5	D	28	0	216 †	901
	L	1414	1172	435	10
Day 6	D	23	9	1235	7
	L	1285	1025	670	452
Day 7	D	38	12	7	5
	L	1189	1040	780	1040
Day 8	D	23	10	6	6
	L	1200	987	1001	976
	D	35	18	9	8

This periodicity of emergence has been observed for other species of cercariae. Cort (1922) records it for *Cercaria elephantis* from *Planorbis trivolvis*, and for an echinostome cercaria from *Physa ancillaria parkeri*. The writer (1931) has observed it for *Cercaria limbifera* from *Lymnaea palustris*, and *Cercaria* 'Z' from *Lymnaea pereger*, and Giovannola (1936) for *Cercaria brevifurca* from *Helisoma trivolvis*, and *Diplostomum flexicaudum* from *Stagnicola emarginata angulata*. This daytime periodicity, however, does not hold good for all cercariae. Under similar conditions *Cercaria cambrensis* 'I' emerges from *Lymnaea truncatula* during the whole 24 hr., but mainly at night (Rees, 1931). Emergence during the day is probably characteristic of those cercariae which exhibit a positive reaction to light, a reaction which seems to exist in most echinostome cercariae.

It is interesting to note the very large numbers of cercariae which emerge from one infected snail. This indicates the enormous reproductive wastage which must occur in the Digenea on account of the difficulties involved in their reaching the final host.

snails were left continuously in the dark the emergence of cercariae practically ceased, a total of only nine emerging during a period of three days and three nights. On returning the snails to normal conditions of light and darkness the periodicity was

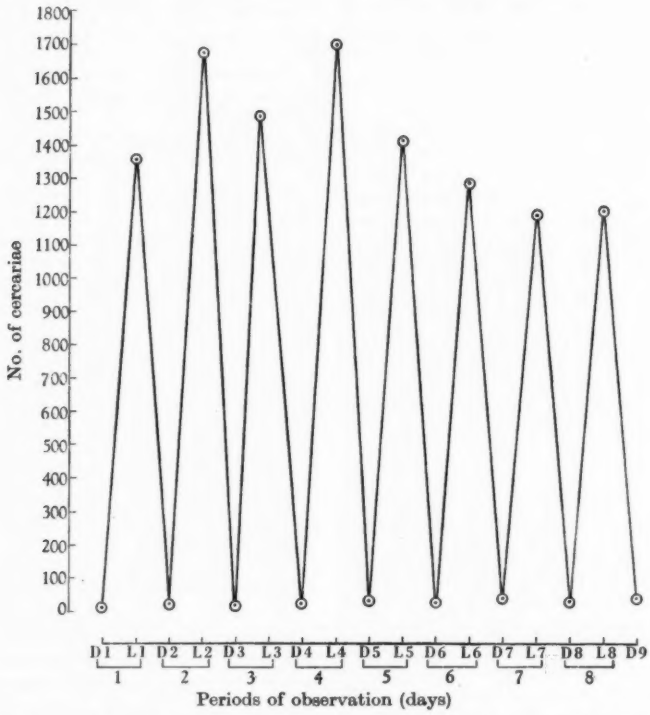

Fig. 1. Average numbers of *Cercaria purpurae* emerging from four specimens of *Nucella lapillus* exposed to alternating periods of 12 hr. darkness (9 p.m.–9 a.m., D) and 12 hr. light (9 a.m.–9 p.m., L), for 8 consecutive days.

Experiment II. An experiment was next performed to find out whether any cercariae would emerge if the snail was kept continuously in the dark, day and night, for a period of several consecutive days. Four infected snails were again taken and exposed at first to normal conditions of 12 hr. darkness during the night and 12 hr. light during the day. They were then placed in the dark for three consecutive days and nights, being thereafter returned to normal conditions. The temperature range was again 15–18° C., and counts of cercariae were made at the end of each 12 hr. period. Exp. I served as control.

The result of this experiment is shown in Table 1, Exp. II, and Fig. 2. It can be seen that at first the periodicity is apparent. Five cercariae emerged during the first night, over 2000 during the following day and seventeen on the second night. When the

again immediately apparent, 1172 emerging on the first exposure to light. The number of cercariae emerging when the snails are kept entirely in the dark is less than when the periods of darkness alternate with light periods. In the latter case, it is very likely that those which do emerge do so in the early part of the dark period, soon after transference from light. When there were no alternating periods of light the maximum number emerging for any 12 hr. period was two.

Light, therefore, seems to be an essential factor for the emergence of *Cercaria purpurae* from *Nucella lapillus*, and under normal conditions there is a marked periodicity of emission which is entirely lost in the absence of light.

The reaction of some other cercariae to complete darkness is slightly different. In the case of *Cercaria limbifera* from *Lymnaea palustris*, a form

which shows a periodicity similar to that of *Cercaria purpurae*, the writer (1931) found that in the absence of light no cercariae emerged for the first 36 hr., and after that they appeared in small numbers during the daytime. Here the periodicity persists even in the absence of light, but the numbers of larvae emerging are considerably reduced.

continuous period of light for four consecutive nights and days. The source of light was a 100 W. bulb kept at a constant distance from the dishes containing the snails. The effect of this was to keep the water in the dishes at a constant temperature of 18° C., otherwise the temperature range was from 15 to 18° C. After exposure to continuous light

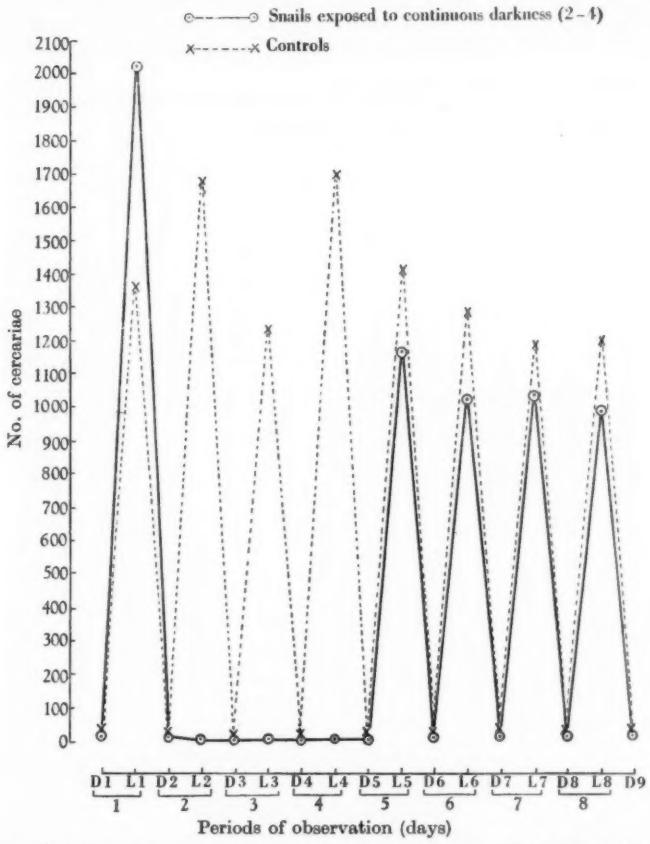

Fig. 2. Average numbers of *Cercaria purpurae* emerging from four specimens of *Nucella lapillus* exposed at first to alternating periods of 12 hr. darkness (D) and 12 hr. light (L), and then for 3 consecutive days (2–4) to continuous darkness, being returned to normal conditions later. Control snails kept under normal conditions.

In the case of *Cercaria* 'Z' Rees, (1931), from *Lymnaea pereger*, which also shows periodicity under normal conditions, cercariae will emerge more or less regularly over the whole 24 hr. in the absence of light, but again the numbers are reduced.

Experiment III. The third experiment was carried out to ascertain the effect of a continuous period of light on the emergence of the larvae. Four infected snails were exposed at first to normal conditions of darkness and light. They were then exposed to a

the snails were returned to normal conditions. Counts were made at the end of each 12 hr. period.

The result of this experiment is shown in Table 1, Exp. III, and Fig. 3. Exp. I served as control. In the early part of the experiment, when the snails were kept under normal conditions, the periodicity of emergence was evident. The period of exposure to continuous light began on the third night following an exposure to light for 12 hr. on the

previous day. Cercariae continued to emerge, and during the first night a total of 1102 cercariae were counted, following a total of 1086 for the 12 hr. period (normal day) immediately preceding it. Thereafter, during the next two 12 hr. periods there was a fall in the number which emerged, in spite of the presence of light, the counts being fifty-three and forty-eight respectively. This initial fall was followed by a gradual rise over the next four 12 hr. periods to a maximum of 1235, after which came to cope with the new situation. Normally, development of the cercariae presumably goes on during the whole of the 24 hr., but during the hours of darkness those which have developed will accumulate inside the snail, to emerge in large numbers on the return of light. When light is continuous there is no such periodicity, and the cercariae probably emerge as soon as they have completed their development. Therefore large numbers appear at first, followed by a gradual falling off while more

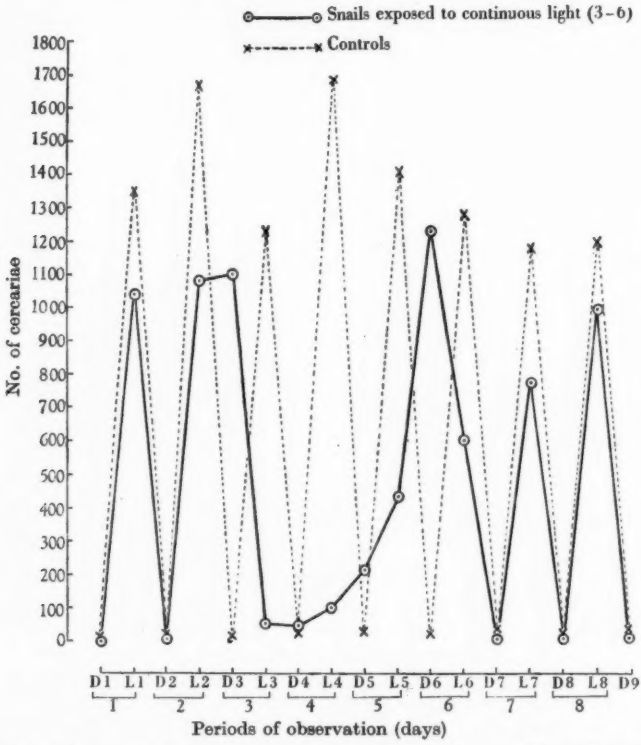

Fig. 3. Average numbers of *Cercaria purpurae* emerging from four specimens of *Nucella lapillus* exposed at first to alternating periods of 12 hr. darkness (D) and 12 hr. light (L), and then for 4 consecutive days (3–6) to continuous light, being returned later to normal conditions. Control snails kept under normal conditions.

a fall to 670 during the following 12 hr. period. The snails were then returned to normal conditions, when the periodicity of emergence was immediately restored.

From this it can be seen that if the infected snails are exposed to continuous light cercariae emerge continuously. The number is high at first, then falls, gradually to rise to a new maximum. The fall in numbers shortly after the beginning of exposure to light may be due to the fact that the rate of development of the cercariae is not increased develop to bring the number up to a maximum again. There must naturally be a cycle in the development of the larvae themselves, as those resulting from one infection will reach the same stage of development at the same time. This cycle is obscured by the alternating periods of light and darkness, but during continuous light it becomes apparent. There is, of course, an added complication in that all the larvae emerging from one host snail may not be the result of one infection, but of several which have taken place at different times.

Experiment IV. As the cercariae normally emerged in large numbers during the light periods coinciding with the daytime, it was decided to find out whether this periodicity could be reversed by exposing the infected snails to 12 hr. darkness during the daytime and 12 hr. light at night. Four snails were again taken and exposed at first to normal conditions of 12 hr. darkness during the night and 12 hr. light during the day. On the second day the periods of darkness and light were reversed, the snails being kept in the dark during the 12 hr. day period, and illuminated with a 100 W. lamp during the 12 hr. night period. The reversal was maintained for a period of four consecutive days, after which the snails were returned to normal conditions. The temperature range was 15–18° C., except for the period of reversal, when it remained constant at 18° C. Counts were made at the end of each 12 hr. period and Exp. I served as control.

The result of this experiment is shown in Table 1, Exp. IV, and Fig. 4. At first the usual periodicity is apparent. On reversal of light and darkness the periodicity is immediately reversed, the larger numbers emerging during the periods of light, now at night, and the smaller numbers during the periods of darkness, now day. On returning to normal conditions the periodicity is again immediately reversed.

The same reversal of periodicity has been observed by Giovannola (1936) for cercariae emerging from three species of snails. In the case of *Diplostomum flexicaudum* from *Stagnicola emarginata angulata* he found that the usual periodicity was still apparent during the first 24 hr. of reversal and thereafter a gradual change took place until the inversion of periodicity was established. In *Cercaria purpurae*, however, inversion is immediate and not gradual.

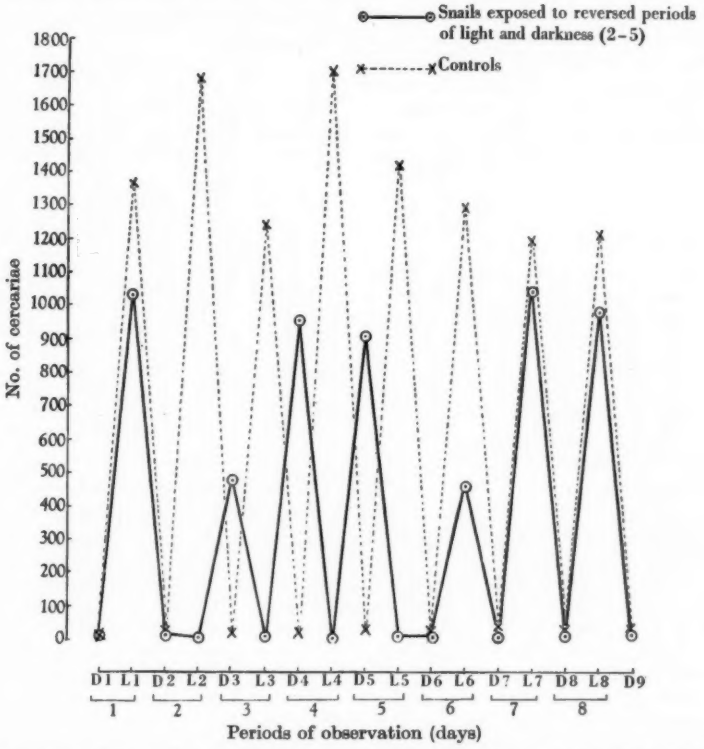

Fig. 4. Average numbers of *Cercaria purpurae* emerging from four specimens of *Nucella lapillus* exposed at first to alternating periods of 12 hr. darkness (D) and 12 hr. light (L), and then to a reversal of the periods of light and darkness for 4 consecutive days (2–5), being returned to normal conditions later. Controls kept under normal conditions.

From these four experiments it can be seen that light is a prime factor in the emergence of *C. purpurae* from its host. This may be accounted for by the fact that the cercariae, in spite of the absence

of any apparent light-sensitive organs, exhibit a markedly positive phototropism. They always collect on the lighter side of the vessel in which they are contained. If the vessel is rotated the cercariae immediately move across to the light side. What the significance of this attraction to light may be is difficult to explain. The cercariae normally encyst around the aperture of the shell of the host snail and on the exposed parts of the body, from where they are taken up by the final host, *Larus argentatus*, when feeding. How the presence or absence of light can be related to the position of the cysts is difficult to imagine. *Nucella lapillus* is usually found near the surface of the water, which may be a partial explanation. On the other hand, the periodicity may be related to the habit of a second intermediate host which has been eliminated from the life cycle during the course of evolution.

TEMPERATURE

While uncovered by the tide the pools in which *Nucella lapillus* lives are exposed to variations in temperature. It was decided therefore to investigate the effect of a decrease and an increase in temperature on the number of larvae emerging from the host snail. A control experiment was carried out at the same time, four infected snails being kept singly in glass dishes at room temperature (15–18° C.), and exposed to alternating periods of 12 hr. darkness and 12 hr. light.

Experiment I. Decrease in temperature. Four infected snails isolated singly in glass dishes were kept under normal conditions of light and at room temperature for a day and a half, and then exposed to temperatures decreasing by 5° every 24 hr. from 15 to 0° C. For temperatures of from 10° C. downwards the dishes containing the snails were surrounded by the required amount of ice, and kept in an oven provided with a glass door, so that they could be illuminated during the daytime. A thermometer was placed in the water and there was no difficulty in maintaining the temperature at a constant level for the required period. More ice was added to bring the temperature down to 0° C. on the fourth day, and during the fourth night the snails were placed in a refrigerator where the temperature was 0° C. After an exposure to 0° C. for 24 hr. the snails were returned on the fifth day to room temperature. Throughout the experiment the snails were subjected to alternating periods of 12 hr. darkness and 12 hr. light as were the controls. Counts of cercariae were made at the end of each 12 hr. period.

The result of this experiment is shown in Table 2 and Figs. 5 and 6. The figures given in the table are the averages for four snails in each case, and the control figures are not represented in the graphs. In Fig. 5 counts of cercariae are given for each 12 hr. period, and in Fig. 6 for each 24 hr. period.

Table 2. *The effect of a decrease in temperature on the emergence of* Cercaria purpurae *from* Nucella lapillus

Duration of experiment 6½ consecutive days. Conditions of light, L, and darkness, D, normal. Figures are averages for four snails in both control and experiment. Experimental snails subjected to decrease in temperature from days 2–4. Counts given for 12 and 24 hr. periods.

Period of observation	Room temp. ° C.	Numbers of cercariae: Control 12 hr.	Control 24 hr.	Exp. I 12 hr.	Exp. I 24 hr.	Temp. for Exp. I ° C.
D	16	24		19		16
Day 1 L	17	1810	1864	1295	1307	17
D	15	54		12		15
Day 2 L	17	2036	2073	7	7	10
D	15	37		0		10
Day 3 L	18	1300	1319	2	2	5
D	16	19		0		5
Day 4 L	17	1295	1307	0	0	0
D	15·5	12		0		0
Day 5 L	18	1107	1122	1253	1258	18
D	16	15		5		16
Day 6 L	18	1143	1163	1047	1057	18
D	15·5	20		10		15·5

It can be seen that there was a rapid fall in the number of cercariae emerging when the temperature was lowered to 10° C., an average of only seven appearing in the water during the 12 hr. light period. No cercariae emerged at night at that temperature. At 5° C. an average of two cercariae emerged during the day and none at night, and at 0° C. emergence ceased completely. Within 2 hr. after the return of the snails to room temperature, cercariae began to emerge, and continued to do so in a more or less steady stream all day, the total number being 1253. Thereafter the normal periodicity was apparent, large numbers emerging during the hours of light and few during the hours of darkness. Compared with the controls at room temperature, it can be seen that a decrease in temperature below 15° C. greatly inhibits the emergence of larvae and below 5° C. emergence ceases altogether. Temperatures as low as these would occur quite frequently, for varying periods, on the shore during the winter months.

Cort (1922) has observed a similar decrease in the number of *Cercaria elephantis* emerging from *Planorbis trivolvis* at decreased temperatures. He kept snails in water from a well at temperatures ranging from 58 to 63° F. for nearly 24 hr. The escape of cercariae was almost completely inhibited.

On return to a high temperature (74° F.) the increase was immediately reflected in the escape of the cercariae. The same is true of *Cercaria cambrensis* 'I', a reduction of temperature to 14° C. greatly reducing the number of larvae emerging from the host snail *Lymnaea truncatula*. It seems probable, therefore, that temperature is an important factor in regulating the escape of cercariae from their snail hosts.

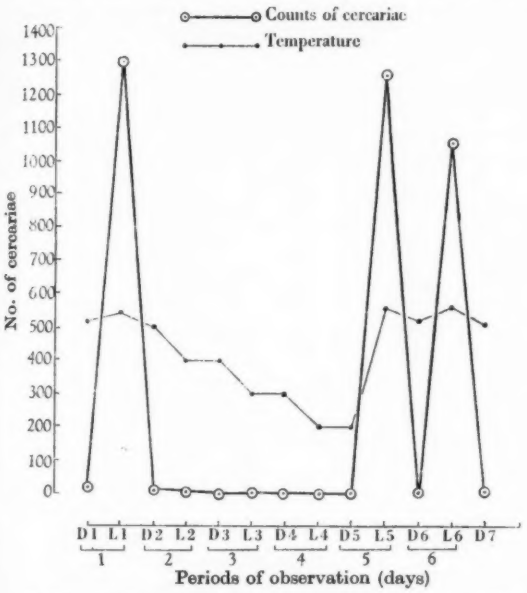
Counts of cercariae
Temperature
No. of cercariae
Temperature (degrees Centigrade)
D1 L1 D2 L2 D3 L3 D4 L4 D5 L5 D6 L6 D7
1 2 3 4 5 6
Periods of observation (days)

Fig. 5. Average numbers of *Cercaria purpurae* emerging from four specimens of *Nucella lapillus* exposed at first to room temperature and then to temperatures decreasing by 5° steps to 0° C., being returned later to room temperature. Counts at the end of each 12 hr. period.

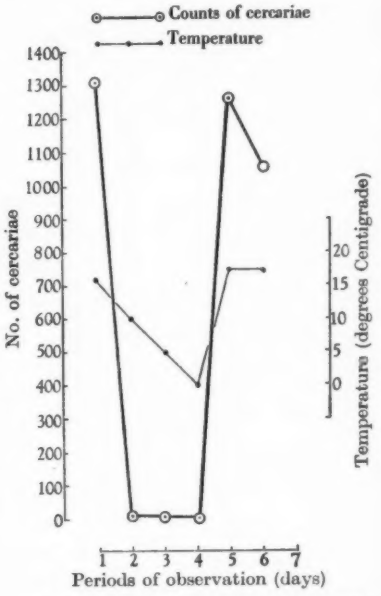

Fig. 6. Average numbers of *Cercaria purpurae* emerging from four specimens of *Nucella lapillus* exposed at first to room temperature and then to temperatures decreasing by 5° steps to 0° C., being returned later to room temperature. Counts at the end of each 24 hr. period.

Experiment II. Increase in temperature. A rise in temperature has a somewhat different effect on the number of larvae emerging. The same controls were used as for the previous experiment, and both controls and experimental snails were subjected to normal conditions of light and darkness. Four infected snails were taken and exposed to an increase in temperature of 1° C. every 12 hr. The dishes containing the snails were kept in an oven where the temperature could be easily controlled and the snails illuminated during the daytime through the glass door. The temperature of the oven was raised 1° every 12 hr., the time taken for the water in the dishes to reach the temperature of the oven after each change being about 1½ hr. Under these conditions of increasing temperature the thermal death-point of *Nucella lapillus* was 31° C. Heat coma sets in at 29° C. Two of the four experimental snails were removed from the oven and returned to room temperature when heat coma became apparent, and the remaining two were exposed to a further increase up to 33° C.

The result of this experiment is shown in Table 3 and Figs. 7 and 8. Under conditions of increased temperature the periodicity of emergence is still apparent, but the number emerging during the day falls gradually up to a temperature of 26° C. The numbers emerging during the periods of darkness, on the other hand, increase gradually up to a temperature of 27° C., so that although the periodicity is still apparent it is not as well marked as in the controls kept at room temperature. Shortly before heat coma sets in, there is a sudden rise in the number emerging during the day, followed by a fall during the following night. In the case of the two snails exposed to a further increase in temperature, there is a fall again at 30° C. and after that no larvae appeared in the water. In the case of the two snails removed from the oven after exposure

Table 3. *The effect of an increase in temperature on the emergence of* Cercaria purpurae *from* Nucella lapillus

Duration of experiment 8 days. Conditions of light, L, and darkness, D, normal. Figures are averages for four snails in both controls and experiment. Experimental snails subjected to gradually increasing temperatures. Two snails (*) returned to room temperature after exposure to 29° C. Counts given for 12 and 24 hr. periods.

Period of observation		Room temp. ° C.	Numbers of cercariae: Control 12 hr.	Control 24 hr.	Exp. II 12 hr.	Exp. II 24 hr.	Temp. for exp. ° C.
	D	16	24	—	14	—	17
Day 1	L	17	1810	1864	1000	1026	18
	D	15	54		26		19
Day 2	L	17	2036	2073	850	893	20
	D	15	37		43		21
Day 3	L	18	1300	1319	350	397	22
	D	16	19		47		23
Day 4	L	17	1295	1307	288	355	24
	D	15·5	12		67		25
Day 5	L	18	1107	1122	202	296	26
	D	16	15		94		27
Day 6	L	18	1143	1163	494	537	28
	D	15·5	20		43		29
Day 7	L	17·5	996	1013	39 137 *	39 147	30 17·5*
	D	15	17		0 10		31 15
Day 8	L	17·5	1038	1052	0 396	0 405	32 17·5
	D	15	14		0 9		33 15

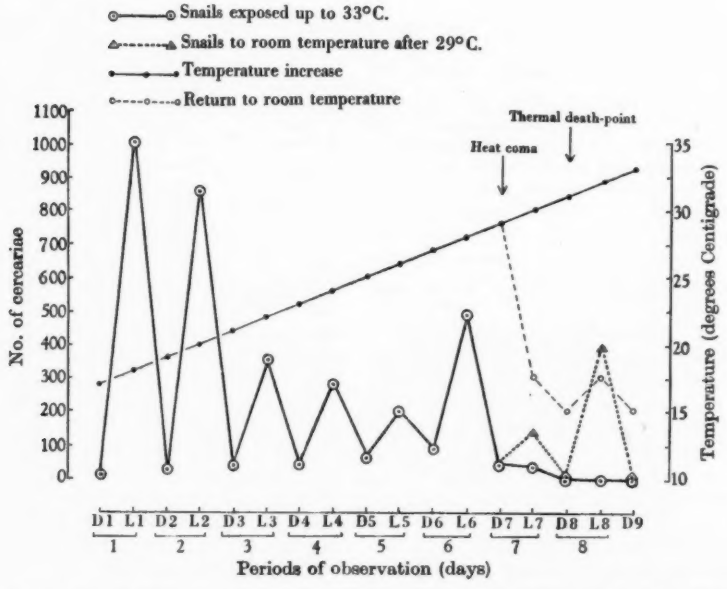

Fig. 7. Average numbers of *Cercaria purpurae* emerging from four specimens of *Nucella lapillus* exposed to temperatures increasing by 1° C. every 12 hr. Two snails returned to room temperature after 29° C. Counts at the end of each 12 hr. period.

to 29° C., recovery took place immediately. On the first day at room temperature 137 cercariae emerged and on the second 396, with small numbers, also, during the hours of darkness.

The sudden emergence of a large number of larvae shortly before heat coma sets in in the host snail is very striking. It is well illustrated in Fig. 8, which shows the number of larvae for each 24 hr. period. The experiment was repeated several times, always with the same result. It is possible that,

SALINITY

As the water in rock-pools is subject to variations in salinity due to evaporation or to precipitation, it was decided to investigate the effect of a decrease and an increase in salinity on the numbers of cercariae emerging from *Nucella lapillus*.

Experiment I. Decreased salinity. Two series of four snails were taken. One series served as controls

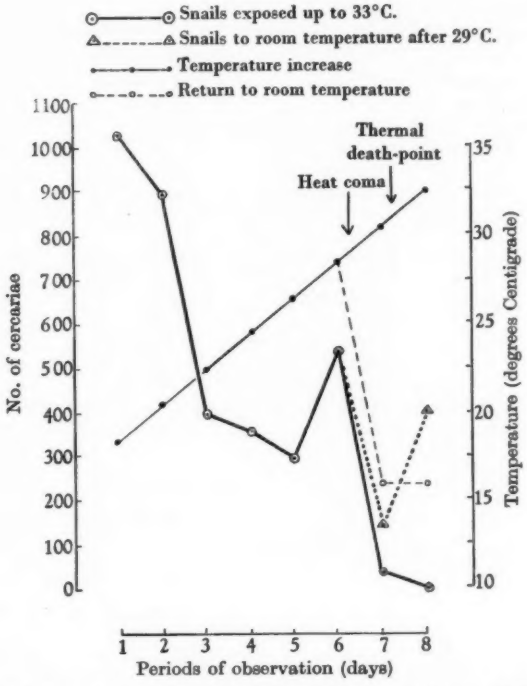

Fig. 8. Average numbers of *Cercaria purpurae* emerging from four specimens of *Nucella lapillus* exposed to temperatures increasing by 1° C. every 12 hr. Two snails returned to room temperature after 29° C. Counts at the end of each 24 hr. period.

although the increase in temperature at first inhibits emergence, it finally stimulates the larvae to develop and they emerge before the snail is finally destroyed. Cercariae emerging at 30° C. die very soon, but below that temperature they live for at least 12 hr., which was the duration of each period of observation.

Increase in temperature therefore inhibits to some extent the emergence of *Cercaria purpurae* from *Nucella lapillus*. Under the conditions in which these two experiments on the effect of temperature have been carried out, the temperature range for the emergence of *Cercaria purpurae* from its host is 10–30° C.

and were kept in normal sea water; the other series were transferred from normal sea water to sea water of gradually decreasing salinity. Normal salinity was taken as that of the water in the bay at the time of collection of the snails, 35·05‰. The volume of the solution in each case was 100 ml. and the series covered the ranges $0{\cdot}875N$, $0{\cdot}750N$, $0{\cdot}625N$, $0{\cdot}5N$, $0{\cdot}375N$ and $0{\cdot}250N$, the sea water being diluted with the requisite amount of tap water. The snails were immersed in each solution for a period of 24 hr. and then transferred to the next. Both the controls and the experimental snails were kept at room temperature and subjected to alternating periods of 12 hr. light during the day

and 12 hr. darkness during the night. Counts of larvae were made at the end of each 12 hr. period.

The results of the experiment are shown in Table 4, Exp. I, and Figs. 9 and 10. Fig. 9 represents the number emerging during each 12 hr. period and Fig. 10 during each 24 hr. period. As the salinity of the water decreases the number of cercariae emerging also decreases, but the periodicity is still apparent. No cercariae emerged when the salinity was reduced to $0{\cdot}375N$ ($13{\cdot}144°/_{oo}$) and after immersion in sea water of this strength for more than 12 hr. the snails died.

Table 4. *The effect of a decrease in salinity on the emergence of* Cercaria purpurae *from* Nucella lapillus

Duration of experiment 7 days. Conditions of light, L, and darkness, D, normal. Figures are averages for four snails in control and Exp. I and for two snails in Exp. II.

Exp. I. Decrease in salinity from N sea water to $0{\cdot}25N$.

Exp. II. Snails returned to N sea water after immersion in sea water of decreasing salinity to $0{\cdot}50N$.

Period of observation		Control (salinity $35{\cdot}05°/_{oo}$) No. of cercariae	Salinity of solution for Exp. I	Numbers of cercariae: Exp. I 12 hr.	Exp. I 24 hr.	Exp. II	Salinity of solution for Exp. II
Day 1	L	360	N ($35{\cdot}05°/_{oo}$)	507	551	444	
	D	12	N ($35{\cdot}05°/_{oo}$)	44		20	
Day 2	L	512	$0{\cdot}875N$ ($30{\cdot}67°/_{oo}$)	302	311	278	
	D	4	$0{\cdot}875N$ ($30{\cdot}67°/_{oo}$)	9		7	
Day 3	L	463	$0{\cdot}75N$ ($26{\cdot}29°/_{oo}$)	205	211	140	As for Exp. I
	D	6	$0{\cdot}75N$ ($26{\cdot}29°/_{oo}$)	6		4	
Day 4	L	480	$0{\cdot}625N$ ($21{\cdot}91°/_{oo}$)	56	66	42	
	D	12	$0{\cdot}625N$ ($21{\cdot}91°/_{oo}$)	10		6	
Day 5	L	473	$0{\cdot}500N$ ($17{\cdot}525°/_{oo}$)	51	53	10	
	D	7	$0{\cdot}500N$ ($17{\cdot}525°/_{oo}$)	2		3	
Day 6	L	678	$0{\cdot}375N$ ($13{\cdot}144°/_{oo}$)	0	0	0	N ($35{\cdot}05°/_{oo}$)
	D	10	$0{\cdot}375N$ ($13{\cdot}144°/_{oo}$)	0		0	N ($35{\cdot}05°/_{oo}$)
Day 7	L	593	—	—	—	0	N ($35{\cdot}05°/_{oo}$)
	D	11	—	—	—	0	N ($35{\cdot}05°/_{oo}$)
Day 8	L	614	—	—	—	0	N ($35{\cdot}05°/_{oo}$)
	D	9	—	—	—	0	N ($35{\cdot}05°/_{oo}$)
Day 9	L	589	—	—	—	71	N ($35{\cdot}05°/_{oo}$)
	D	6	—	—	—	3	N ($35{\cdot}05°/_{oo}$)
Day 10	L	574	—	—	—	176	N ($35{\cdot}05°/_{oo}$)
	D	7	—	—	—	7	N ($35{\cdot}05°/_{oo}$)

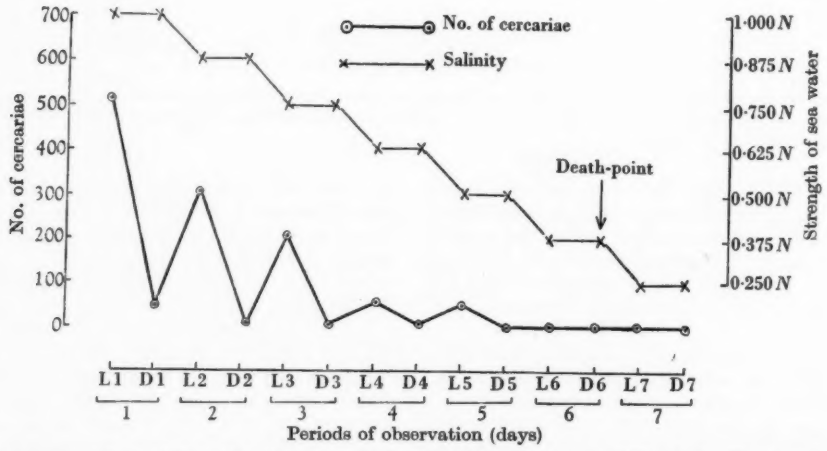

Fig. 9. Average numbers of *Cercaria purpurae* emerging from four specimens of *Nucella lapillus* immersed in sea water of gradually decreasing salinity from N to $0{\cdot}25N$. Counts at the end of each 12 hr. period.

As, under the conditions of the experiment, the snails die after immersion in sea water of a strength of $0{\cdot}375N$, it was decided to find out whether recovery would take place after immersion in sea water of a strength of $0{\cdot}5N$ ($17{\cdot}525‰$). The experiment was therefore repeated with two other snails. one appeared in the water, three emerged the following night and 176 on the fifth day. The periodicity was again evident, though the numbers at first were comparatively small. Recovery after exposure to decreased salinity takes longer than in the case of decreased and increased temperature.

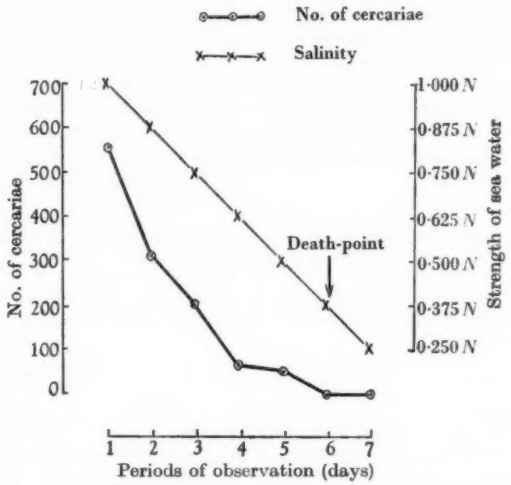

Fig. 10. Average numbers of *Cercaria purpurae* emerging from four specimens of *Nucella lapillus* immersed in sea water of gradually decreasing salinity from N to $0{\cdot}25N$. Counts at the end of each 24 hr. period.

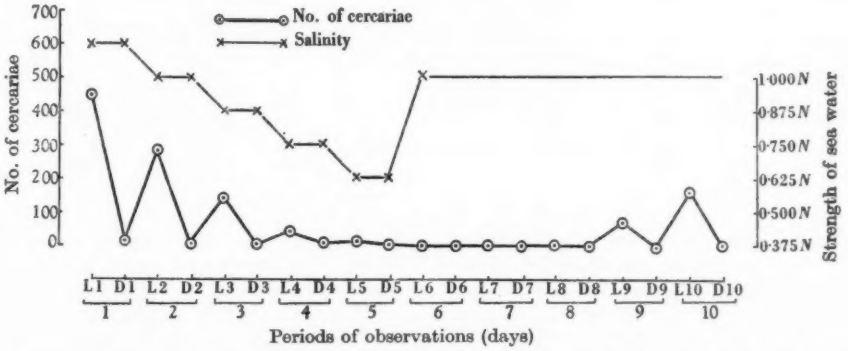

Fig. 11. Recovery of *Nucella lapillus* and reappearance of cercariae on return of snail host to N sea water after immersion in sea water of gradually decreasing salinity from N to $0{\cdot}50N$.

It was conducted as before, the snails being transferred at intervals of 24 hr. to sea water of decreasing salinity until it reached $0{\cdot}50N$. After immersion in this for 24 hr. the snails were returned to normal sea water ($35{\cdot}05‰$). The result of this experiment is shown in Table 4, Exp. II, and Fig. 11. After return to normal sea water no cercariae emerged from the host snail for a period of three days and three nights. On the fourth day seventy-

Possibly the effect on the host snail is more drastic, and therefore the recovery period must be longer.

In addition to affecting the host snail, a decrease in salinity will affect the activity and longevity of the cercariae themselves. Stunkard & Shaw (1931) carried out some experiments on the effect of dilution of sea water on *Cercaria sensifera*, a species now regarded as being synonymous with *C. purpurae*. They found that in tap water the cercariae

died within an hour. In sea water diluted to $0{\cdot}125N$ they lived for 3 hr., and at $0{\cdot}25N$ there was little ill effect, and they lived almost as long as in greater concentrations, although they were usually less active after the first few hours. At a strength of $0{\cdot}5N$ they appeared active and normal for almost, if not quite, as long as in undiluted sea water. It seems, therefore, that the cercariae can survive a slightly greater dilution than can the host snail. During rain, it is of course possible that the cercariae which have emerged may be exposed to further dilution of the sea water in small pools. Large numbers of them can overcome this unfavourable condition by encysting. The effect of dilution on the cysts is not known.

$1{\cdot}50N$. The same controls were used as for the previous experiment. The temperature range during the experiment was from 16 to 20° C.

Four infected snails were taken and were kept at first in normal sea water. They were then transferred at intervals of 24 hr. to sea water of increasing strength. The host snails seemed to react more rapidly, at first, to increased than to decreased salinity, and so one snail was returned to N sea water after immersion for 24 hr. in $1{\cdot}125N$ ($39{\cdot}43°/_{00}$). The remaining three gradually adjusted themselves and were transferred to $1{\cdot}25N$ and later to $1{\cdot}375N$. After immersion for 24 hr. in $1{\cdot}375N$ sea water another snail was removed and returned to N sea water, while the remaining two were

Table 5. *The effect of an increase in salinity on the emergence of* Cercaria purpurae *from* Nucella lapillus

Duration of experiment 7 days. Conditions of light, L, and darkness, D, normal. Experimental snails exposed to gradually increasing salinities. Snail 1: returned to N sea water after immersion in $1{\cdot}125N$. Snail 2: returned to N sea water after immersion in $1{\cdot}375N$.

Period of observation		Control (salinity $35{\cdot}05°/_{00}$) No. of cercariae	Salinity of solution for exp.	Number of cercariae: Exp. 12 hr.	Exp. 24 hr.	Snail 1 12 hr.	Snail 1 24 hr.	Snail 2 12 hr.	Snail 2 24 hr.
Day 1	L	403	N ($35{\cdot}05°/_{00}$)	780	785	—	—	—	—
	D	5	N ($35{\cdot}05°/_{00}$)	5					
Day 2	L	692	$1{\cdot}125N$ ($39{\cdot}43°/_{00}$)	62	62	—	—	—	—
	D	6	$1{\cdot}125N$ ($39{\cdot}43°/_{00}$)	0					
Day 3	L	394	$1{\cdot}25N$ ($43{\cdot}81°/_{00}$)	47	51	326	342	—	—
	D	7	$1{\cdot}25N$ ($43{\cdot}81°/_{00}$)	4		16			
Day 4	L	308	$1{\cdot}375N$ ($48{\cdot}19°/_{00}$)	1	1	450	461	—	—
	D	10	$1{\cdot}375N$ ($48{\cdot}19°/_{00}$)	0		11			
Day 5	L	417	$1{\cdot}50N$ ($52{\cdot}575°/_{00}$)	0	0	354	356	199	203
	D	6	$1{\cdot}50N$ ($52{\cdot}575°/_{00}$)	0		2		4	
Day 6	L	502	—	—	—	231	231	531	533
	D	7	—	—	—	0		2	
Day 7	L	483	—	—	—	353	355	482	486
	D	5	—	—	—	2		4	

Increased salinity, Experiment II. To discover the effect of an increase in salinity on the number of cercariae emerging, infected snails were placed in solutions of sea water of gradually increasing strengths from normal to $1{\cdot}50N$. The salinity of the water in the bay was again $35{\cdot}05°/_{00}$. One litre of a $2N$ solution was made up as follows: 750 ml. of normal sea water was measured, and this contained 26·28 g. salt. The amount of salt required for 1 l. of a $2N$ solution was 70·10 g., and 43·82 g. of Tidman's sea salt was therefore dissolved in a small amount of tap water and the solution added to the 750 ml. of normal sea water. The resulting solution was made up to 1 l. with tap water. From this $2N$ solution the series of solutions necessary for the experiment were made up by dilution with tap water. The range selected was $1{\cdot}125N$, $1{\cdot}250N$, $1{\cdot}375N$, and

subjected to a further increase to $1{\cdot}50N$, when death of the snails took place. Counts of larvae were made at the end of each 12 hr. period.

The results of this experiment are shown in Table 5, and Figs. 12 and 13. The controls behaved normally, as can be seen from the table. On exposure to sea water of increased salinity there was a rapid fall in the number of cercariae emerging from the host snails. The periodicity was still apparent at strengths of $1{\cdot}125N$ and $1{\cdot}25N$. An average of only one cercaria emerged during the daytime at a salinity of $1{\cdot}50N$, and thereafter no cercariae appeared in the water. In the case of snail 1, returned to N sea water after immersion in $1{\cdot}125N$, recovery was immediate and the normal periodicity with large numbers was restored. Snail 2, returned to N sea water after immersion in $1{\cdot}375N$,

showed the same immediate recovery. No recovery took place and no cercariae emerged after immersion for 24 hr. in sea water with a salinity of 1·50*N*.

Increased salinity, therefore, inhibits the emergence of cercariae from *Nucella lapillus*. Recovery takes place up to a strength of 1·375*N*, but not beyond. The salinity range therefore within which the larvae can emerge from the host snail under the conditions of the experiments is 0·50–1·375*N*.

of encystment but do not seem to have any adverse effect on the cercariae themselves.

In its natural environment *N. lapillus* can escape from an unfavourable salinity between tides by crawling out of the pools. Many specimens are found attached to bare rocks or under seaweed. In such circumstances any changes in salinity would not affect them. They may, of course, be exposed to the direct rays of the sun, but the thickness of

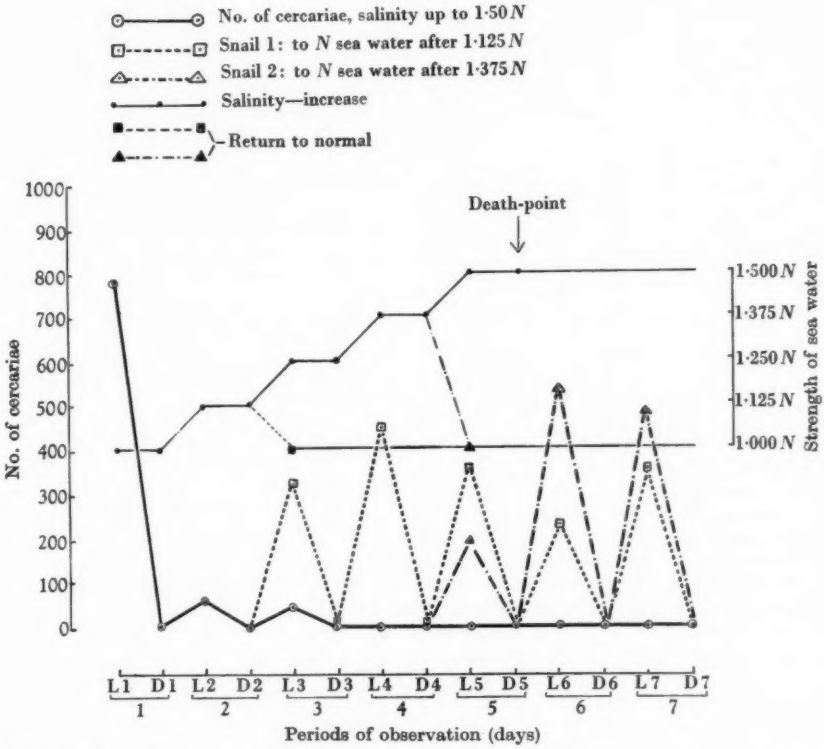

Fig. 12. Numbers of *Cercaria purpurae* emerging from *Nucella lapillus* immersed in sea water of gradually increasing salinity. Counts at the end of each 12 hr. period. Snail 1 returned to *N* sea water after immersion in 1·125*N*. Snail 2 returned to *N* sea water after immersion in 1·375*N*.

An increase in salinity will also affect the larvae themselves. The writer (1937) has made some experiments to ascertain the effect of an increase in salinity on the larvae. As in the case of decreased salinity, the range seems to be a little wider for the cercariae than for the host snail. When immersed in sea water of twice normal strength the cercariae immediately become sluggish and die after a few hours without any attempt at encystment. Increasing salinities of up to 1·75*N* accelerate the rate

the shell or the covering seaweed would offer some protection.

From the experiments described in the present paper, it can be seen that light, temperature and salinity are important factors in the control of the emergence of *Cercaria purpurae* from *Nucella lapillus*. Like the majority of echinostome cercariae, this species emerges from its host snail in the presence of light, emergence being greatly retarded in the hours of darkness. A distinct diurnal

periodicity is therefore apparent, which can be reversed immediately on the reversal of the periods of light and darkness. An increase and a decrease in temperature inhibit emergence, as likewise do an increase and a decrease in salinity. The temperature range and the salinity range for emergence are fairly wide, so that the species is adapted to withstand the changes in the physical environment which must occur in an intertidal zone at different times of the year and on different individual days.

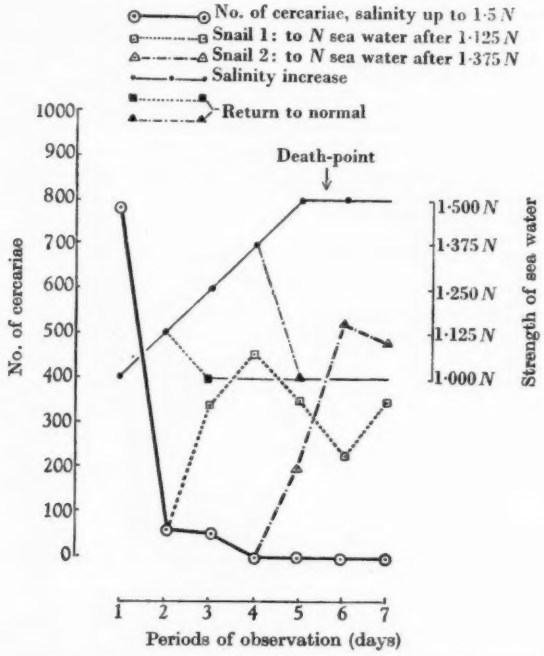

Fig. 13. Numbers of *Cercaria purpurae* emerging from *Nucella lapillus* immersed in sea water of gradually increasing salinity. Counts at the end of each 24 hr. period. Snail 1 returned to *N* sea water after immersion in 1·125 *N*. Snail 2 returned to *N* sea water after immersion in 1·375 *N*.

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ON TWO NEW SPECIES OF TICK

By FRANK A. TURK, Ph.D., F.R.E.S., F.Z.S.

(With 11 Figures in the Text)

Both species of tick which are described in the present paper are members of insular faunas and demonstrate remarkably well the tendency, shown by these parasites in geographical isolation, to species formation. In this connexion it is perhaps worth quoting what Cooley & Kohls (1945) say concerning this subject in their recent work on the genus *Ixodes*: 'These studies', they write on p. 1 of their introduction, 'have revealed in considerable measure the more useful specific characters and the importance of a real appreciation of the significance of geographical range and variation in the differentiation of species....In determination of species limits we have considered not only morphologic but also biologic factors such as host relationships and geographic distribution. No subspecies are recognized.' There is little doubt but that geographical distribution will be found to play an almost equally important role in the genus *Amblyomma*.

The type of the female and larva and syntypes of the nymphs are in my own collection.

Ixodes dorrien-smithi n.sp.

This is an exceptionally small species of tick. All the specimens so far taken have come from the Isles of Scilly shrew, *Crocidura cassiteridum* Hinton, 1924, six specimens of which were examined, four from the island of St Mary's and two from the island of Tresco. From these individuals were taken one larva (August 1945), fourteen nymphs (April and May 1946) and one female (June 1946). Only one specimen of the host was found to be without ticks in some stage or other of their development and that one had been dead for some considerable time.

This form appears not to be nearly related to any other species of tick and occupies, if the sum total of the characters are taken together, a distinctly isolated position. The only other species of tick which is found on shrews in Europe appears to be *Ixodes trianguliceps* Birula, 1895 (=*I. tenuirostris* Neumann, 1901) from which *I. dorrien-smithi* differs in many characters and can at once be separated by the presence of the coxal spines which are not present in *I. trianguliceps*. Perhaps more nearly related is *I. arvicolae* Warburton, 1926 (?=*I. apronophorus* Schulze, 1924), but from this it is distinguished by the characters of the rostrum, the dorsal shield and the tarsi.

The present new species I have named after Major C. Dorrien-Smith, the Lord Proprietor of the islands, who has been to great trouble to provide me with specimens of the shrew from Tresco and who has on many occasions provided me with much information about the zoology of these islands.

Female. Body rather elongate; the shields are medium brown in preserved examples and the other parts a creamy white when ungorged. The anal groove is only very slightly divergent behind, whilst the genital one is rather more so and with no sinuations or indentations. Length of the single female 2·2 mm.

The dorsal scutum is rather elongate (Fig. 1). The scapulae are well marked but slim. The lateral carina is very apparent and reaches the sides just posterior to the lateral angles which are rather rounded. The cervical grooves are not so well developed and become very shallow posteriorly, hardly reaching the lateral borders of the scutum. Posteriorly, the scutum is bluntly rounded, its greatest width being behind the anterior third. The punctuations are relatively sparse and shallow and somewhat difficult to see.

The structure of the capitulum is better seen (Fig. 2 dorsal, and Fig. 3) in the text-figures than described. The dorsal ridge is well developed and conspicuous cornua are present. The areae porosae, on the single specimen of the female so far known, are obscure and not well defined, discontinuous and suboval. The second and third articles of the palps are subequal in length and carry very few rather conspicuous setae. They reach a little beyond the hypostome which is rather elongate and armed with 2+2 files of eleven teeth, the lateral series of which are very much larger than the median. The auricula on the underside of the capitulum are rather strongly developed.

The coxae (Fig. 4) are very characteristic of the species: the first is armed with an external and internal spine, both of which are almost equally developed; the second has an internal spine very much broader but not so highly chitinized as the external one which is somewhat conical; the third has both spines present, the external being rather more strongly developed than the internal; the fourth has a fairly well-marked conical external

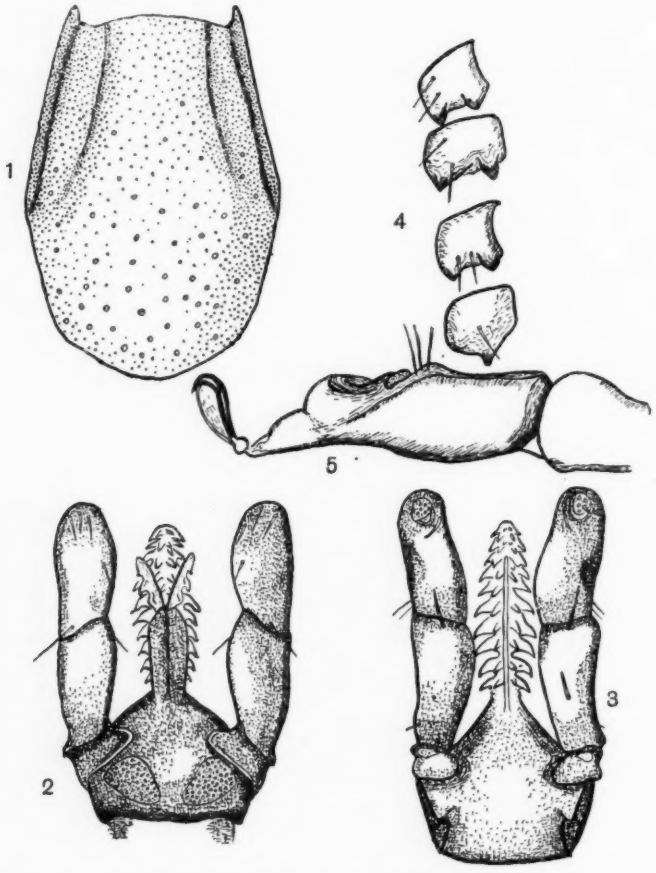
spine, and the internal spine is represented by a mere tubercle which is, however, easily seen on the single type specimen.

The tarsus of the first leg (Fig. 5) terminates abruptly without tapering, and the organ of Haller is set rather far from the distal end. The very characteristic form of the tarsus is best made out from the figure, and this, together with the condition of the coxae, provides a good and easily seen set of characters by which this form may be differentiated from others of the same genus.

The plate of the spiracle is nearly completely circular, the macula fairly centrally placed and the goblets few.

Nymph. The nymph is very like the female, but the internal spurs on coxae III and IV appear to be reduced or absent on most of the specimens and, although this character is variable, in no case are they so well developed as in the female. In all the spirit-preserved material which I possess the unengorged nymphs are reddish in colour and broadly rounded behind, the greatest breadth being 0·65 mm. and the total length 0·85 mm. The gorged nymphs are more elongate and whitish with a total length of 1·6 mm.

Larva. The larvae are without spurs to coxae III and IV, and the dorsal scutum is shorter and more broadly rounded posteriorly. The single



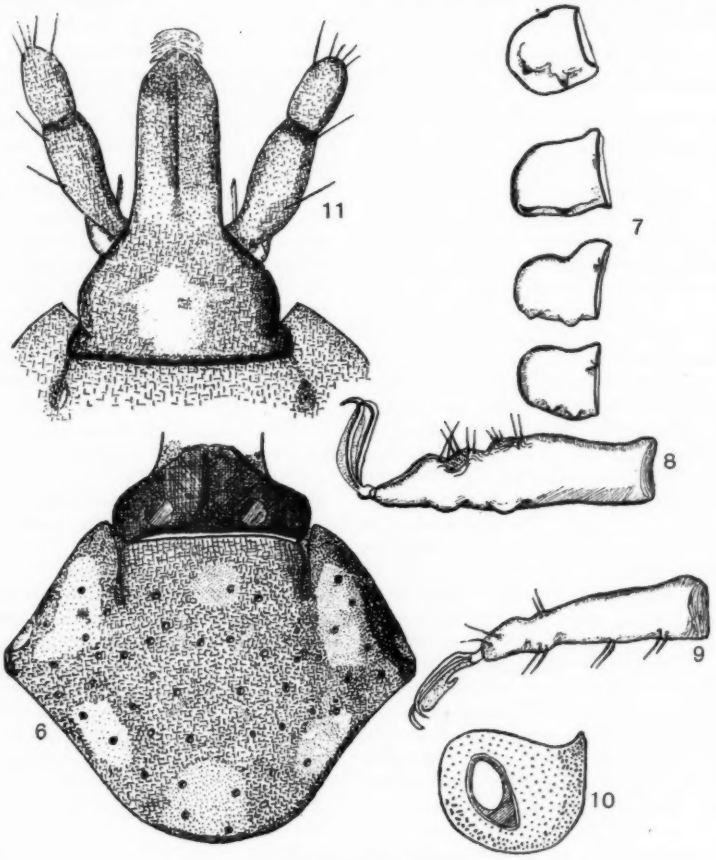
Figs. 1–5. *Ixodes dorrien-smithi* n.sp.

Fig. 1. Dorsal scutum of female.
Fig. 2. Capitulum of female, dorsal.
Fig. 3. Capitulum of female, ventral.
Fig. 4. Coxae of legs I–IV, right side, female.
Fig. 5. Tarsus I of female.

specimen which I possess is 0·92 mm. long and 0·53 mm. greatest breadth.

All the type material, as listed above, is at present in my own collection.

The species is undoubtedly closely allied to *Amblyomma torrei* Vigueras, 1934, which species was redescribed more fully by Dr Whittick (1939). *A. torrei* has occurred on *Cyclura macleayi* in Cuba



Figs. 6–11. *Amblyomma trinitatis* n.sp.

Fig. 6. Dorsal scutum and basis capituli of female.
Fig. 7. Coxae I–IV of female, right side.
Fig. 8. Tarsus I of female.
Fig. 9. Tarsus IV of female.
Fig. 10. Spiracular plate of female.
Fig. 11. Capitulum and anterior of dorsal scutum of nymph.

Amblyomma trinitatis n.sp.

The material for which this species is diagnosed was sent to me by Mr R. G. Donald, B.Sc., formerly of the Imperial College of Tropical Agriculture, Trinidad. It consists of a single female, four nymphs and eight larvae, all of which were taken on a ground lizard at St Augustine, Trinidad, 7 October 1945. The type material is preserved, both as microscopic preparations and in alcohol, and is at present in my own collection.

and on *C. macleayi caymanensis* in Little Cayman Island, West Indies. It is unfortunate, in the circumstances, that the genus and species of the host of the present species were not determined. There is little doubt, however, but that *Amblyomma scutatum* Neumann, 1899, *A. cruciferum* Neumann, 1901, *A. torrei* and the present species form a nearly related group whose speciation has been brought about by geographical isolation; in the case of the last three by their being confined to islands, either in the Greater or Lesser Antilles. It is interesting

that all the insular species should have much reduced ornamentation on the dorsal scutum and that there should be sparse white hairs on the dorsum, both of which characters are lacking in the mainland form which has a fairly wide distribution in Central and South America. Like the other two insular forms, *A. trinitatis mihi* has ornate nymphs; the nymph of *A. scutatum* does not appear to be known.

Female. Length 2·5 mm., greatest breadth 1·7 mm. This is considerably smaller than the dimensions given by Whittick for *A. torrei*. The dorsal scutum (Fig. 6) is far less shouldered than in any of the other species, and this serves to distinguish it at once from all other members of the group. The cervical grooves are very deep but do not extend very far posteriorly, and distally they are very slightly incurved. The scapulae are pointed, their lateral margins being slightly sinuate. In the only specimen of the female which I possess the capitulum is pressed backwards so that the anterior part is wedged between the scapulae, but this is hardly likely to be its natural appearance. I have, however, drawn it in Fig. 6 as it is in the specimen itself. The punctuations on the dorsal scutum are well marked and moderately numerous although hardly so many as in *A. torrei*. The ornamentation is obscure and difficult to make out, the areas for the most part not being well defined. Of all the ornamentation marks the ocular spot is by far the best defined.

The basis capituli is broad and somewhat wedge-shaped with a central depression. The areae porosae are circular but obscure, difficult to make out, and disappeared when the specimen was mounted. The hypostome is short and somewhat slim with a dentition of 3 + 3, the palps extending beyond its distal margin. The general shape of the whole body of the female is broadly oval with well-defined festoons. There are a few sparsely distributed white body hairs on the dorsal side of the body posterior to the scutum.

The coxae (Fig. 7) show decided similarities to those of *A. torrei*: the first has an internal and an external spur almost equally well developed; the second has two reduced spurs which are little more than well-marked tubercles; the third has two very short blunt spurs; and the fourth two spurs similar to those of the third but placed nearer together.

Tarsus I tapers abruptly and has two rounded spurs ventrally as shown in Fig. 8. Tarsus IV tapers gradually and has one slight ventral spur which is little more than a broadly rounded protuberance (Fig. 9). The spiracular plate (Fig. 10) is like that of *A. torrei* but gently rounded dorsally.

Nymph. Length 1·75 mm. In general it shows the characters of the female, but the capitulum is very different. The hypostome is longer and stouter and the palps more slender. The dorsal scutum is pointed anteriorly, rather more sharply than in the female, and the punctuations on it are very much fewer. The ornamentation is difficult to make out in the specimens preserved in spirit, but again the ocular spot is well marked, and also the limiting spot, in some specimens.

Larva. Length 0·65 mm. The larva is similar to the nymph in most respects, but the punctuations are very sparse indeed on the dorsal scutum. The dorsal scutum has a small, anterior median and a larger posterior median depression on it which are easily seen in a few of the specimens. The dorsal scutum is rather rounder in the larva, but in this stage also it shows traces of ornamentation. Ornate larvae have not, I think, been recorded for any other species in the genus.

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THE TRANSMISSION OF *SPIROCHAETA DUTTONI* NOVY & KNAPP BY *PEDICULUS HUMANUS CORPORIS* DE GEER

By R. B. HEISCH, M.B., B.Chir. (Cantab.) and P. C. C. GARNHAM, M.D. (Lond.), D.P.H.
From the Division of Insect-Borne Diseases, Medical Research Laboratory, Kenya

Nicolle's original theory (Nicolle & Anderson, 1927*a*) that tick-borne spirochaetes are gradually changed into louse-borne ones by adaptation in the new host has been widely accepted (Chandler, 1940; Strong, 1945). Hindle (1931) divides the process into five steps, as follows:

(1) Spirochaete cycle in rodents in which the vector is the *Ornithodorus* tick.

(2) Accidental transmission by tick to man.

(3) Endemic form of the tick-borne disease in man.

(4) Accidental transmission by louse to man.

(5) Complete adaptation of tick infection in the louse.

The only experimental evidence hitherto in support of the last two steps (i.e. transmission of the tick-borne spirochaete by the louse) is that provided by the work of Nicolle & Anderson (1926) on *Spirochaeta hispanica*. They found that whilst the infection is normally transmitted by the tick *Ornithodorus marocanus*, in the laboratory it could also be carried by lice. The reverse process (i.e. the transmission of the louse-borne form of the disease by ticks) was shown to be possible by both Manteufel and Neumann (quoted by Wenyon, 1926).

Brumpt (1927) and Craig & Faust (1943) state that the tick-borne *Spirochaeta duttoni* is not infective for the louse; our work, however, produces evidence to the contrary.

In regard to nomenclature, the name *S. duttoni* is employed here for the spirochaete responsible for the well-known relapsing fever of East Africa, carried by *Ornithodorus*; the name *Spirochaeta recurrentis*, for the spirochaete recently described by us (Garnham, Davies, Heisch & Timms, 1947), responsible for the first outbreak of louse-borne relapsing fever known to have occurred in Kenya.

MATERIAL AND METHODS

The lice used in the transmission work were obtained from a clean area (Nairobi). They were first fed on infected animals and then twice a day on ourselves; in the intervals they were kept in an incubator at 28° C. (relative humidity of approximately 75%). There was always a high mortality and it was difficult to keep sufficient alive for the experiments.

The presence of spirochaetes in lice was demonstrated either in Leishman stained smears or by dark ground illumination of saline emulsions. The emulsion was also used for infecting animals; these included white mice of a mixed strain, white rats, and monkeys (*Cercopithecus aethiops johnsoni* Pocock, *C. mitis albotorquatus* Pousargues, and *C. mitis kolbi* Neumann).

During the experiments it was found necessary to test the transmissibility of the East African strain of *Spirochaeta duttoni* by ticks, and nymphs from laboratory colonies of *Ornithodorus moubata* and *O. savignyi* were fed on infected monkeys. After their meal the ticks were kept in an incubator at 28° C. for 3 weeks, and then at 37° C. for varying periods. Coxal fluid, faeces, and emulsified contents were examined and the ticks were refed on clean mice or monkeys. The most certain way of determining the infectivity of ticks was by feeding them on animals, and the following technique was devised for testing individual ticks. Mice were immobilized on their backs by strapping their extended limbs with elastoplast and fastening tape across their throats. The abdomens were then shaved or the hair removed by a depilatory agent. Ticks were placed individually on the abdomens and held under glass capsules which were removed when the ticks began to feed. After feeding they were put in test tubes, and ticks and mice were numbered separately. Blood slides were taken daily from the mice. It is interesting to note that no spirochaetes were found in the coxal fluid and excreta of three ticks subsequently proved positive by this method. The above technique not only shows the proportion of infective ticks in a given batch but the actual ticks concerned, and facilitates the tracing of the infection in succeeding generations.

TRANSMISSION EXPERIMENTS

There were seven successful experiments which are described below; of these the last four (Exps. 4, 4A, 4B and 4C) were of particular interest in that the *duttoni* spirochaetes were passed through four successive batches of lice.

Experiment 1. Nearly 300 lice (L6B) were fed twice daily for 2 days on monkeys heavily infected

with *duttoni*. Sixteen days after the original feed, it was found that there had been a high mortality amongst the lice and only eleven remained alive; six of these showed spirochaetes.

Experiment 2. Approximately 150 lice (L 7) were fed for 2 days on monkey 55, heavily infected with *duttoni*. Sixteen days after the original feed, five of these lice were examined and one contained spirochaetes. On the 17th day, the thirteen lice remaining were emulsified with 2 c.c. of saline and injected intraperitoneally into monkey 179, which subsequently became infected. A drop of the emulsion, examined by dark ground illumination, revealed a few sluggishly moving spirochaetes.

Experiment 3. Nearly 150 lice (L 8) were fed twice daily for 4 days on monkey 12, heavily infected with *duttoni*. Fifteen days after the original feed, five lice were examined and one contained spirochaetes. On the 18th day, twenty-five lice remained; these were emulsified with 2 c.c. of saline, and a drop examined by dark ground illumination showed fairly numerous spirochaetes which appeared dead or dying. The infection, nevertheless, was successfully transmitted to monkey 178.

Experiment 4. Approximately 600 lice (L 11) were fed for 4 days on monkey 191, showing a moderate infection of *S. duttoni*. Fourteen days after their original feed five were examined and two were positive, spirochaetes being scanty in one and numerous in the other. On the 15th and 16th days two lots of ten lice were examined individually but all were negative. On the 17th day a further ten were examined and found negative, and thirty were emulsified with saline and inoculated into monkey 263 which became infected. On the 18th day thirty-two lice remained, ten were negative on examination and the other twenty-two were inoculated into monkey 271 which also became infected.

Experiment 4A. About 400 lice (L 12) were fed for 3 days on monkey 263 which had been infected by *duttoni* passaged once through lice (Exp. 4). On the 4th, 5th, 6th and 8th days after the original feed, batches of ten lice were examined individually but all were negative. On the 9th day thirty-five remained; these were emulsified with saline and injected into mice (345 and 346) which developed the infection. No spirochaetes were seen in a drop of the emulsion examined by dark ground illumination.

Experiment 4B. Approximately 1000 lice (L 16) were fed for 3 days on monkey 426 infected with *duttoni* passaged thrice through lice. On the 4th day after their original feed thirty lice were found dead; these were inoculated into mice 444 and 445, and one (mouse 444) became infected. On the 5th day thirty-five lice were found dead or dying and were inoculated into mice 446 and 450; one (mouse 446) became infected. On the 6th, 7th and 8th days fifty-eight lice died or were found moribund; they were inoculated into mice 446, 450–457, 470–471, with negative results. On the 10th day five lice were examined and one was positive. The remainder were inoculated into monkey 507 and mouse 508, both of which developed the infection.

Experiment 4C. The previous experiment showed that lice were being infected as early as the 5th day. In the present experiment batches of lice (L 17) were inoculated daily into mice from the beginning in order to determine when they first became infective. The results are shown in Table 1.

Table 1. *Early development of* Spirochaeta duttoni *infection in lice*

Date	No. of lice inoculated into two mice	Results	Remarks
1st day	—	—	360 lice (L 17) fed on infected monkey 492
2nd day	30	Both mice became infected	—
3rd day	30	Negative	—
4th day	30	Negative	—
5th day	30	One mouse became infected	—
6th day	30	Negative	—
7th day	30	Both mice became infected	—

It will be noted that the lice first became infected on the day following their infective feed, and although they were negative on the 3rd, 4th and 6th days, on the 5th and 7th they were positive. It must be emphasized that in this experiment proof of infection depended solely on the results in animals and no spirochaetes were actually seen in the lice themselves.

The results of the above experiments are summarized in Tables 1 and 2, and two negatives are also included. The latter are not described in detail because, first, the batch of lice (L 6A) all died during the first ten days after being fed on a *duttoni* case, and secondly, because the lice (L 10) were fed on a patient with only a few spirochaetes in his blood. It is perhaps worth noting that although it has been easy to transmit *S. duttoni* to lice from infected monkeys, we have not yet succeeded from human cases. Spirochaetes were not seen in the lice until the 15th day, but the presence of the infection was demonstrated by inoculations into animals as early as the 2nd day. It is unlikely that this early infection was due to spirochaetes persisting from the original feed, and it seems probable

that this and the other infections during the first ten days were caused by the organism in an invisible stage.

This cycle appears to differ from that of the recently isolated Kenya strain of *recurrentis* in which a definite negative phase was observed. In both, however, the negative cycle in the louse is terminated by the appearance of metacyclic forms about the 15th day. These forms are characterized by a permanent corkscrew shape and a slender appearance.

DISCUSSION

The foregoing experiments incriminated the louse as a laboratory vector of *S. duttoni*. Two criticisms can be levelled at these findings; first, were the lice used in the tests 'clean', and secondly, was the strain of spirochaete really *duttoni*? With regard to the first criticism, it should be noted that the lice were not obtained from a laboratory colony but were collected from prisoners' blankets in Nairobi. The only louse-borne relapsing fever in Kenya occurred recently as an epidemic 250 miles away, and it was most improbable that the infection could have been conveyed to Nairobi. If an accidental infection of the lice by *S. recurrentis* were responsible for our results, then the disease in inoculated animals would have been of the well-defined *recurrentis* type. This, however, was never the case and it therefore appears justifiable to assume that the lice used in the experiments were 'clean'.

The evidence that the spirochaete was really *duttoni* is as follows: The strain was obtained from a patient living in one of the well-known *duttoni* areas near Nairobi and we have shown already that this particular organism behaves in a very different way to *S. recurrentis* with which it has no cross-immunity. Also its pathogenicity to animals is similar to that of the *S. duttoni* described by Nicolle & Anderson (1927*a*). Its transmissibility by *Ornithodorus* ticks provided further proof of identity. The detailed results are shown in Table 6. Two species of *Ornithodorus* (*moubata* and *savignyi*) became infected, though the proportion found positive was small.* Hereditary transmission of the infection was obtained with *O. moubata*. Out of a batch of thirty-eight eggs, nineteen (50 %) were proved to be infected by the technique described on p. 247.

It should be noted that in Exps. 1 and 3 the monkeys had been infected with *recurrentis* 2–3 months previously. It was first thought that the

Table 2. *Transmission of* Spirochaeta duttoni *by lice*

Batch no. of lice	Date of first infective feed	Date spirochaetes* first demonstrated	Remarks
L 6 A	8. ii. 46	No infection	All lice died before 10th day
L 6 B (Exp. 1)	17. ii. 46	17th day	Six lice of 11 positive
L 7 (Exp. 2)	19. iii. 46	17th day	—
L 8 (Exp. 3)	19. iii. 46	16th day	—
L 10	30. iii. 46	No infection	Fed on human case with scanty infection
L 11 (Exp. 4)	10. iv. 46	15th day	—
L 12 (Exp. 4 A)	8. v. 46	10th day	Spirochaetes not seen but transmission in animals
L 16 (Exp. 4 B)	22. vi. 46	5th day	Spirochaetes not seen but transmission in animals
L 17 (Exp. 4 C)	9. vii. 46	2nd day	For details of Exp. 4 C, see Table 1

* Spirochaetes were demonstrated either direct or by animal inoculation.

THE COURSE OF LOUSE-TRANSMITTED *SPIROCHAETA DUTTONI* INFECTIONS IN ANIMALS

Our previous work provided a clear picture of the reaction of experimental animals to ordinary *S. duttoni*. Table 3 gives the results of louse-transmitted *duttoni* infections in animals; it will be noted that they behaved exactly like ordinary *duttoni*, even after the spirochaetes had been passaged four times through lice. The infections in monkeys proved fatal, except in the case of the more resistant species, *Cercopithecus mitis kolbi*; in white mice the disease was persistent, with irregular relapses. *Spirochaeta recurrentis* produces a totally different effect and is thus easily distinguished.

Cross immunity tests were performed in order to confirm these conclusions. Animals which had recovered from the louse-borne *duttoni* disease were inoculated a month or so later with *S. recurrentis* and all showed normal infections with the latter species. On the other hand, when inoculated with a tick-borne *duttoni* strain no infection resulted. Cross-immunity between the louse-borne and the tick-borne *S. duttoni* was thus demonstrated. Table 4 summarizes the results which also show that there was no antigenic difference between the first and third louse-transmitted infections (mouse 335).

Table 5 traces the course of the original *S. duttoni* infection through the various animals and lice used in the experiments.

* Hindle (1911) has pointed out that negative results (or low infectivity) in many transmission experiments may be due to an active immunity in the tick itself.

Table 3. *Pathogenicity of louse-borne* Spirochaeta duttoni *in animals*

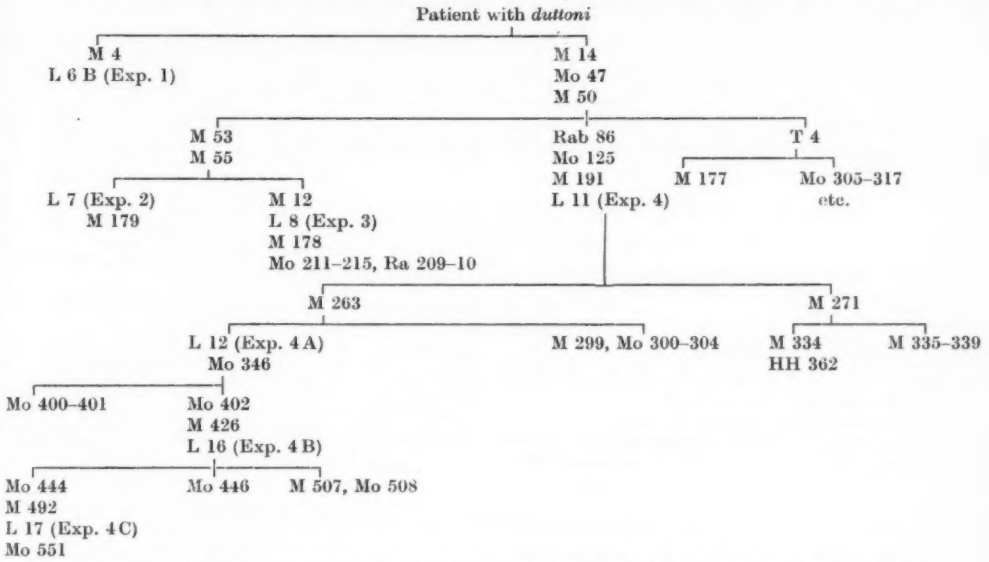
Serial no.	Animal	Source of infection	Results of daily blood examinations: 2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	Remarks
178	Monkey	Lice (Exp. 3)	–	–	–	–	+	+	+	+	Died	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	—
214	Mouse	M 178	+	+	+	–	–	–	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	4 other mice reacted similarly
210	Rat	M 178	+	+	+	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	1 other rat reacted similarly
263	Monkey	Lice (Exp. 4)	–	–	–	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+	–	–	–	+	+	+	–	—
299	Monkey	M 263	+	+	+	+	+	+	+	Died	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	—
302	Mouse	M 263	+	+	–	–	–	+	+	+	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	4 other mice reacted similarly
271	Monkey	Lice (Exp. 4)	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+	+	+	+	+	+	Died	.	.	.	.	—
334	Monkey	M 271	–	–	+	+	+	+	+	+	+	Died	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	5 mice also inoculated and reacted typically
362	Hedgehog	M 334	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	.	.	.	.	—
346	Mouse	Lice (Exp. 4A)	–	–	–	–	–	–	–	+	+	–	–	–	–	–	–	–	+	+	+	+	+	Sacrificed	.			Second passage through lice
345	Mouse	Lice (Exp. 4A)	–	–	–	–	–	–	–	–	Sacrificed	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	Second passage through lice
369	Monkey	Mo 345	–	–	–	+	+	+	+	Died	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	—
446	Mouse	Lice (Exp. 4B)	–	–	–	–	+	+	+	+	+	–	+	+	–	–	–	+	+	+	+	+	–	–	–	–	–	Third passage through lice
551	Mouse	Lice (Exp. 4C)	–	–	–	–	–	–	–	+	+	+	+	+	+	–	–	+	+	+	+	+	–	–	–	–	–	Fourth passage through lice

Table 4. *Cross-immunity: louse transmitted* Spirochaeta duttoni

Serial no.	Animal	Date of onset	Length of infection*	Date of reinfection	Species of reinoculation	Remarks
209	Rat	12. iv. 46	3 days	9. v. 46	*S. recurrentis*	Typical *recurrentis* infection; no cross-immunity
210	Rat	12. iv. 46	15 days	9. v. 46	*S. recurrentis*	Typical *recurrentis* infection; no cross-immunity
211	Mouse	12. iv. 46	10 days	9. v. 46	*S. recurrentis*	Typical *recurrentis* infection; no cross-immunity
211	Mouse	12. iv. 46	10 days	25. v. 46	Tick-transmitted *S. duttoni*	No infection; cross-immunity
213	Mouse	12. iv. 46	11 days	9. v. 46	*S. recurrentis*	Typical *recurrentis* infection; no cross-immunity
213	Mouse	12. iv. 46	11 days	25. v. 46	Tick-transmitted *S. duttoni*	No infection; cross-immunity
214	Mouse	12. iv. 46	23 days	9. v. 46	*S. recurrentis*	Typical *recurrentis* infection; no cross-immunity
214	Mouse	12. iv. 46	23 days	25. v. 46	Tick-transmitted *S. duttoni*	No infection; cross-immunity
335	Mouse	17. v. 46	22 days	11. vii. 46	Louse-transmitted *S. duttoni* (3rd passage)	No infection; cross-immunity

* Infection = louse-transmitted *S. duttoni* (first louse passage).

Table 5. *Diagram showing passage of* Spirochaeta duttoni *through mammals, lice and ticks*



M, monkey; Mo, white mouse; Ra, white rat; Rab, rabbit; HH, hedgehog; L, louse; T, *O. moubata*.

Table 6. *Transmission of* Spirochaeta duttoni *by* Ornithodorus moubata *and* O. savignyi

Batch number	Species	Date of first infective feed	Date examined	Results	Results of feeding progeny of infected ticks
T 2 B	*O. moubata*	8. ii. 46	5 weeks later	Emulsified contents of ten inoculated into monkey 38—*negative*	—
T 3 B	*O. savignyi*	8. ii. 46	5 „	Coxal fluid *negative*. Batch fed on monkey 72—*negative*	—
T 4	*O. moubata*	2. iii. 46	5 „	Coxal fluid of 1 tick *positive*. Batch fed on monkey 177—*positive*	Monkey 185—*negative*
			10 „	13 ticks fed on 13 mice—3 *positive*	Mice 418 and 422—*positive*
T 5	*O. savignyi*	2. iii. 46	5 „	Coxal fluid of 1 tick *positive*. Batch fed on monkey 232—*negative*	—

lice might have developed the infection, either because the *duttoni* spirochaetes had become modified through contact with the immune bodies of the earlier infection, or else because the *duttoni* strain had reactivated the *recurrentis* and caused it to reappear in the blood. There was no evidence to support either of these assumptions; and of the animals used in the remaining five transmissions, none had been infected with *recurrentis*. It was therefore concluded that the experiments represented the simple carriage of *S. duttoni* by lice.

In view of these facts, an epidemic of louse-borne *duttoni* relapsing fever must be regarded as a possibility. It is strange that such outbreaks have rarely if ever occurred and this may perhaps be explained by the existence of an immunity against *duttoni* in the population.

The instability of strains of spirochaetes has been stressed by many workers. This, however, has not been our experience. Repeated passage through different animals left the antigenic constitution of the spirochaete apparently unchanged, and even four transmissions through an abnormal intermediate host failed to cause mutation. In other

words, although the results demonstrate that the louse can act as a vector of *S. duttoni*, no evidence was obtained that the *recurrentis* type of relapsing fever was being evolved in this way.

SUMMARY

1. Cyclical development of *Spirochaeta duttoni* in lice was observed in seven experiments. In the last four, the same strain was transmitted through four successive batches.

2. The resultant infections in animals behaved exactly like *S. duttoni* even after the fourth transmission through the abnormal host.

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NUTRITIONAL REQUIREMENTS OF FLEA LARVAE, AND THEIR BEARING ON THE SPECIFIC DISTRIBUTION AND HOST PREFERENCES OF THE THREE INDIAN SPECIES OF *XENOPSYLLA* (SIPHONAPTERA)

By M. SHARIF, D.Sc. (Panjab), Ph.D. (Cantab.), F.N.I., B.M.S.(I).
Assistant Director in Charge of Department of Entomology, Haffkine Institute, Parel, Bombay (India)

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I. INTRODUCTION

It is now known that stored agricultural products (see, for instance, Buxton, 1932, p. 291; Hirst, 1927*a*, p. 405), such as cereals, pulses, fodder, cotton, etc., serve as convenient vehicles for the transport of rats and their fleas from one place to another even at a great distance. The possibility of transport of *Xenopsylla cheopis* (Rothschild) by ships absolutely free from rats was hinted at by Hirst (pp. 318, 420). The Advisory Committee (1908, p. 255) suggested that the larvae of fleas, 'since they can feed upon almost any kind of organic rubbish, and pupae, which require no food, could be carried considerable distances in merchandise, *i.e.* for periods as long as one or two months'. The Committee actually found (p. 241) a number of the larvae of this rat-flea 'in the sacking' in the neighbourhood of a 'nest made by *M. rattus* on a grain bag'. In view of the fact that the 'factors governing transference of the species of fleas from place to place are still imperfectly understood' (Hirst, 1933, p. 96), it was considered necessary to ascertain whether the larvae of *X. cheopis*, *X. brasiliensis* (Baker) and *X. astia* Rothschild can breed successfully on the flour of cereals alone. As the stored grains, especially those of wheat, rice, millets, barley, etc., are attacked by insect pests, such as the larvae and the adults of beetles, *Sitophilus granarius* (Linnaeus), *S. oryza* (Linnaeus), *Tribolium confusum* Duval, *T. ferrugineum* (Fabricius), *Oryzaephilus surinamensis* (Linnaeus), *Rhizopertha dominica* (Fabricius) and *Sitodrepa panicea* (Linnaeus), the larvae of moths, *Sitotroga cerealella* (Olivier) and *Ephestia kuehniella* Zeller, etc., small quantities of the excrement and detritus that they produce are always present. Eggs are laid by fleas indiscriminately in the stored products, when rats infested with fleas visit them. Larvae hatched from such eggs possibly may have no food supply other than the flour dust (Bacot, 1914, p. 513; Hirst, 1927*a*, pp. 397, 404), as owing to the frequent shifting of the stored products, the larvae of fleas are usually forced to live in an environment, in which there is little chance of the presence of flea and rat faeces.

The findings presented here are based on experimental work done in the laboratory and on about 3 years' field experience gained in an inquiry into the recrudescence of plague in the districts of Sholapur and Dharwar in the Bombay Province. The observations made provide a reasonable explanation for the host preferences and the irregular and patchy distribution of the three species of rat-fleas in India. An account of the ecological conditions governing the burrows and nesting conditions of both wild and domestic rodents in these districts will be published later; but a few of the observations bearing on the present problem have been incorporated in this paper.

II. METHODS AND TECHNIQUE

The methods employed for conducting the experiments were similar to those described in an earlier communication (Sharif, 1937, p. 225). The different diets used were given in a finely powdered form, thoroughly mixed with 5 g. of ignited and acid-washed fine quartz sand. In the case of a simple diet

only 1 g. was used; for a mixed diet ½ g. of each of the constituents was thoroughly mixed before the addition of the sand. In these experiments Merck's 'Yeast medicinal dry powder', dried horse blood and highly milled wheat flour devoid of bran were used.

Every possible precaution was taken to avoid any other organic substance coming in contact either with sand or food. Sable's hair brushes and Petri dishes, utilized for examination of the larvae, were always thoroughly washed with soap and water. Brushes were sterilized with absolute alcohol, and Petri dishes were flamed. In no case was the same brush or Petri dish used for two different diets.

Only completely unfed and recently hatched larvae less than 24 hr. old were employed. The larvae were reared in specimen tubes which were placed in a desiccator, containing an atmosphere of 80 % relative humidity (R.H.) controlled by a mixture of extra pure sulphuric acid (Merck) and water in a proportion given by Buxton & Mellanby (1934, p. 174). The desiccator was placed in a dark cupboard in an air-conditioned room maintained at 25° C. (77° F.) ± 1° C. All the experiments were conducted under parallel conditions.

By breeding the three Indian rat-fleas on different diets but under otherwise similar conditions, I have attempted to isolate the nutritional factors and to study their effect statistically. For assessing the nutritive values of the diets the criteria adopted were: (1) the duration of the active larval life and that of the combined larval and pupal life for both sexes, (2) mortality rate in the larval stage, (3) proportion of cocoon formation or pupation, (4) proportion of success in rearing of adults, (5) regularity or irregularity in emergence of adults of both sexes, and (6) variation in the size of adults. Most of these features were statistically evaluated, and were subjected to suitable tests of significance based on the formulae given by Fisher (1941) and Fisher & Yates (1943). In order to ensure the accuracy of results, the experiments of each type were repeated for the number of times entered in the column marked 'Experiments tried'. The results of all the experiments of a type were pooled. The means given are weighted averages. The tests of significance used were the t and χ^2, using 'Yates' correction for continuity' except when the numbers were too few and the exact method of χ^2 was used. When tested in pairs for significance, a result of comparison which is not statistically different is denoted by the sign of $-$, that which is significant at 5 % level is indicated by $+$, and that at 1 % level by $\times$. The signs in each column of the tables indicate the level of significance between a particular value, against which a sign is inserted, and the one higher in the column with which it is connected by an arrow.

Undoubtedly, the 'best criterion for assessing the suitability of a diet is the length of larval life, *i.e.* the time from hatching to pupation' (Fraenkel & Blewett, 1943*b*, p. 459); but in fleas whose larvae spin cocoons, it is very difficult to ascertain the exact duration of the complete larval stage. Consequently, only the active larval life up to the time of cocoon formation has been taken into consideration. In cases where the larvae formed naked pupae, the duration of larval life up to the resting larval stage is assessed, as the time taken for cocoon formation approximates to that.

III. HISTORICAL SURVEY

Since the time of Leewenhoeck (1683, p. 78), many workers have tried to rear flea larvae under experimental conditions. The diets used by them have been mentioned elsewhere (Sharif, 1937, pp. 226–7). From the results of Bacot (1914, pp. 513–33), Webster (1930, pp. 398–403) and Sikes (1931, pp. 246–8), it appears that the methods employed by them in rearing flea larvae were not very successful, as a fairly high and variable mortality occurred in their experiments. In 1937 I described a standardized food for flea larvae, consisting of dried horse blood and yeast, which has been very successful, as almost 100 % success was obtained in rearing fleas from larvae to adults. Further, this food has the advantage that its quantity and quality can be gauged exactly. On consideration of my findings, Buxton (1937, p. 12) suggested that yeast 'presumably supplies accessory food factors, one may suppose that under natural conditions micro-organisms serving the same purpose occur in fragments of bedding or of rat's dung'.

In view of the different role of species of flea in the epidemiology of plague (Hirst, 1923, p. 817), the question of the distribution of three species of *Xenopsylla* Glinkiewicz in India has gained great importance. Consequently, many workers (see, for instance, Cragg, 1921, 1923; Hirst, 1926, 1927*a*, *b*, 1933; Sharif, 1930) have given detailed records of their distribution in India, Burma and Ceylon. Buxton (1941) has mapped their distribution in the world. In spite of these attempts the irregular distribution of these rat-fleas still defies reasonable analysis.

IV. EFFECTS OF DIFFERENT DIETS ON THE DEVELOPMENT OF THE LARVAE OF THE THREE INDIAN RAT-FLEAS

In order to determine the comparative nutritional requirements of the larvae of the three Indian rat-fleas, their recently hatched and unfed larvae were reared on (*a*) blood alone, (*b*) highly milled wheat flour devoid of bran, and mixed diets in equal parts of (*c*) blood and wheat flour, and of (*d*) blood and yeast. The differences in their development on these diets

were noted. A mixture of blood and yeast was used for control experiments, as it possesses all the essential requirements of an ideal food for flea larvae.

Sikes (1931, p. 245) has found that different kinds of food have different hygroscopic properties, and the optimum atmospheric humidity for rearing flea larvae varied according to the kind of food employed so as to satisfy their water requirements. The degree of moisture content of the different diets tried, when in equilibrium with 80 % R.H. at 25° C., has been assessed roughly by noting the physical changes in them and the behaviour of flea larvae when fed on them. It was observed that whenever the water content of a food was so low that it just permitted flea larvae to grow, the food particles adhered to the larvae and the pupae, and the fully grown defaecated larvae failed to spin cocoons, perhaps because they could not afford to lose water through secretion of silken threads for spinning cocoons (Sharif, 1948). Appreciable increase in volume of a dry diet at 80 % R.H. denotes its high water content. Even variation in the colour of diets, especially those that contained blood, indicated different proportions of water. If the colour of the food was dull red, its water content was low; but if it was bright red, the water content was high.

(*a*) *Blood.* Dried horse blood alone was completely unsuccessful as a larval diet for *X. astia* and *X. brasiliensis* (Table 1); most of the larvae died in the first instar, though 3·9 % of them in the former species reached the third instar, and 3 % in the latter formed small naked pupae after 29–42 days, from which no adults emerged. It was also very unsuitable for the larvae of *X. cheopis*, but in this species 3·3 % larvae bred successfully into small but strongly sclerotized adults; most of them died in the second instar. The fact that the average duration of the resting larval and pupal life of this species (Table 2) on blood alone was not statistically different from that on a mixed diet of blood and flour, or blood and yeast, suggests that the slight success in rearing adults was due to a favourable change that occurred in the dried blood on prolonged keeping at 80 % R.H.

Only a few larvae of the three species grew slowly and irregularly on blood alone, which was unsuccessful in bringing about pupation and cocoon formation. A significantly lower proportion of the dead larvae of *X. cheopis* than that of *X. astia* on this food (Table 1) indicates the simpler nutritional requirements of the former species. In *X. brasiliensis* 160 larvae died in different active instars in 13–55 ($25{\cdot}31 \pm 0{\cdot}8319$) days, in *X. cheopis* 145 larvae in 13–49 ($32{\cdot}48 \pm 0{\cdot}6965$) days and in *X. astia* 178 larvae in 9–58 ($31{\cdot}32 \pm 0{\cdot}9215$) days. The fact that only the average period taken by the larvae of *X. brasiliensis* to die on this food was significantly shorter than that taken by the other two species, shows that blood is most unsuitable for the larvae of this flea.

Throughout the exposure of 70 days to 80 % R.H., the blood remained dry, dull red and discrete; it did not swell appreciably, as its moisture content was very low (see Sikes, 1931, p. 244). The blood particles adhered to the larvae in large numbers, especially to the dead ones; this hindered the growth of fungus on the dead larvae, which normally occurs at this humidity.

(*b*) *Wheat flour.* Highly milled wheat flour devoid of bran is deficient if used as the sole diet, as only 27 % of larvae in *X. cheopis*, 42·9 % in *X. brasiliensis* and 20·5 % in *X. astia* were reared into adults, and these after very long intervals (Tables 1, 2). This food gave rather better success with the larvae of *X. brasiliensis*; the average active larval life of this flea was statistically the shortest, though it was significantly longer than that of *X. cheopis* when reared on the mixed diet of blood and yeast.

When larvae are fed on flour, there is a considerably longer active larval life, and a significantly higher mortality rate in the larval stage and lower proportion of cocoons formed in *X. astia* than in the other two species (Tables 1, 2): this might be attributed either to insufficient moisture in the meal in equilibrium with 80 % atmospheric humidity, or to its comparatively low nutritive value for the larvae of this species. The latter alternative appears to be more reasonable, as the fact that some larvae lived a long time and spun cocoons demonstrates the presence of enough moisture in this food at this humidity.

The growth of flea larvae on flour was irregular, as every developmental stage from the first larva to the adult were found after 41 days in *X. cheopis* and *X. brasiliensis*, and from the second larval instar to the adult after 60 days in *X. astia*. There were 10·5 % lagging larvae in *X. cheopis*, 8·2 % in *X. brasiliensis* and 15 % in *X. astia*; they lived for 45–63, 40–55 and 72–91 days respectively, and did not form cocoons or pupae. When flea larvae (Table 2) were nourished on an ideal diet of blood and yeast, all the adult females emerged first, and then 2 days after the males started emerging; but when they were reared on flour alone, the adults of both the sexes emerged indifferently. This fact and a marked disparity in the developmental rate of flea larvae on flour indicate that some favourable change had occurred in it, which permitted successful breeding of some of the larvae. The only appreciable change observed was that the dead larvae and flour had slight fungous growth. It is suggested that highly milled wheat flour without bran, by itself, is an insufficient diet for flea larvae, and that association of micro-organisms, such as fungi or perhaps yeast or bacteria, with it was responsible for the successful growth of some of them.

The general behaviour of flour exposed to the atmospheric humidity of 80 % denotes that it absorbed more water than the dried blood. The former adhered to the larvae, but not to such an extent as the latter. The flour definitely increased in volume; but it looked slightly moist and discrete after 141 days' exposure to this humidity. If not disturbed for a number of days, it formed a loosely held brittle mass with sand, and a thin crust was formed on the surface. When the larvae were present in the tube, the surface of the food and sand mixture was always covered with a large quantity of frass in the form of fluffy flour. The larvae fed on this food were sluggish, and only those that remained within cocoons bred successfully. The ability of some larvae to spin cocoons shows that flour at 80 % R.H. contains enough moisture to permit of cocoon formation. The cocoons formed by the larvae fed on flour were smaller and softer, having looser meshes, than those spun by larvae fed on the mixed diet of blood and yeast; this seems to show that the former food had less moisture content than the latter (Sharif, 1948).

(c) *Blood and wheat flour.* On this mixed diet (Tables 1, 2) 54·4 % larvae of *X. cheopis* and 48·4 % of *X. brasiliensis* pupated, and the remainder died in the resting stage after completion of their active larval life. In contrast, only 58·2 % of the larvae of *X. astia* completed their active larval life, and 40·7 % of them pupated. Only 21·3 % larvae of *X. cheopis*, 9·7 % of *X. brasiliensis* and 24·2 % of *X. astia* were reared into adults. The addition of blood to flour resulted in a considerable shortening of the combined larval and pupal life of the three species.

A statistically higher mortality rate in the larval stage and longer average active larval life on this mixed diet in *X. astia* (Tables 1, 2) than in the other two species, indicate that its larvae have more particular nutritional requirements; moreover, the food, at 80 % R.H., may give a less favourable atmospheric condition. But a significantly shorter resting larval and pupal life in *X. astia* than in the other two species, suggests that those of its larvae which became adults were able to obtain more nutritive food owing to their prolonged larval life; possibly the nutritional value of the food improved with the length of exposure to this humidity.

The proportions of adults of *X. cheopis* and *X. astia* (Table 1), when their larvae were reared on the mixed diet of blood and flour, were not significantly different from their proportions when reared on flour alone; similarly, no statistical difference existed in rearing of adults of both these species on the former diet. On the other hand, a significantly lower proportion of the larvae of *X. brasiliensis* reached the adult stage on this mixed diet than when they were fed on flour alone, and also than when the larvae of *X. cheopis* and *X. astia* fed on the former diet. This shows a more pronounced desiccating influence of the mixed diet on the pupae of *X. brasiliensis* than on those of both the other species, as the proportion of its pupation did not differ statistically from that of either of the other two species on this diet, and also from that when its larvae fed on flour alone. A marked desiccating influence of the mixed diet on the pupae of *X. cheopis* and *X. astia* is also demonstrated by the fact that in spite of the better growth of their larvae on it (in comparison with flour alone), there was no improvement in successful rearing of the adults.

There was a little cocoon formation on this mixed diet, and a fairly high mortality occurred in the pupal stage owing to loss of water. Adults of both the sexes emerged indifferently and after great difficulty from the pupal skins, and they were less active after emergence. These facts bear testimony to the dryness of this food.

Evidently, the nutritive value of this mixed diet is higher for the larvae of the three species than that of flour alone; but the desiccating influence of the former diet, kept at 80 % R.H., was responsible for a high rate of mortality.

The mixed diet of blood and flour increased slightly in bulk at 80 % R.H.; but it remained dry and discrete throughout an exposure of 86 days, and was dull red in colour. It adhered to the larvae and the pupae so largely that they looked almost covered with it. The larvae were very sluggish, and a large quantity of food was passed by them undigested.

(d) *Blood and yeast.* The combined larval and pupal life of the three species on the mixed diet of blood and yeast was much shorter than on flour alone or the mixed diet of blood and flour (Table 2), mainly due to a statistically great reduction in their active larval life. Evidently, the first diet has a much better nutritive value for flea larvae than the other two. The larval growth was regular on this mixed diet, as the interval between the first and the last cocoon formation was only 4–5 days. The percentage of cocoon formation was very high, and there was almost 100 % pupation and adult emergence (Table 1).

This mixed food seemed to gain more water than the others from the atmospheric humidity of 80 %; it did not adhere to the larvae and the pupae and was of bright red colour.

The dimensions (average lengths and breadths) of the adults of the three species (Table 3) reared from the larvae on blood and yeast were statistically much greater than those of the adults from the other diets.

The adults of *X. cheopis* and *X. astia* (Table 3), whose larvae fed on the mixed diet of blood and flour, were statistically longer and broader than those reared on flour alone; this indicates the better nutritive value of the former food. The fact that the breadth measurements of both the sexes of *X. brasiliensis* showed significantly inconsistent disparity on

these two diets and no statistical difference existed in their length measurements confirms the previous conclusion (see p. 256) that addition of blood to flour has not resulted in improvement in rearing of its larvae.

The average length of a few adults of *X. cheopis* (Table 3), obtained from the larvae reared on blood alone, was not statistically different from that of those whose larvae were nourished on flour alone or the mixed diet of blood and flour. Only their average breadth was significantly less than that of adults from larvae on a mixture of blood and flour. These facts indicate that the larvae of this species that bred successfully on blood alone may have had an opportunity of obtaining a small amount of an accessory food substance.

It is, therefore, evident that the condition of the adults of the three Indian rat-fleas, as expressed by their length and breadth measurements, is also a good indication of the efficiency of the larval diets, and they confirm my conclusions based on the duration of larval life or combined larval and pupal life, and on the proportions of larvae which became pupae or adults.

V. DIETETIC REQUIREMENTS OF FLEA LARVAE

It is now known that the food available to flea larvae consists of varied organic matter; but there is still no information regarding the actual food which they select. In order to throw light on their exact nutritional requirements, an attempt will now be made to collect and combine all the available information on the subject.

Blood is generally considered an integral part of the larval food for almost all the fleas. But my recent experiments on *X. cheopis*, *X. brasiliensis* and *X. astia* (Table 1), and earlier ones (Sharif, 1937, p. 231) on *Nosopsyllus fasciatus* (Bosc) show that sterile or pure, dried horse blood is an inadequate diet for the larvae of these species; on blood alone successful rearing of adults is not possible. Evidently blood lacks some important factor necessary for normal and successful breeding of them, which is compensated by the addition of yeast, or possibly when blood is contaminated with micro-organisms. The meagre success obtained in rearing flea larvae on blood alone (see p. 255) is attributed to the association of a few micro-organisms with blood due to a chance contamination, which increases its nutritive value by providing vitamins of the B group, as in *Rhodnius prolixus* Stål (Wigglesworth, 1936, p. 289; Brecher & Wigglesworth, 1944, p. 224). Sikes (1931, p. 247) obtained partial and highly variable success in rearing flea larvae on blood at 80 and 90 % R.H. The contradictory results obtained on this or other diets by different workers may be due to the fact that flea larvae have never been reared on sterile food.

My work also shows (Tables 1, 2) that highly milled wheat flour without bran is an unfavourable larval diet for the three Indian species of *Xenopsylla*. There is a high mortality and great irregularity in development; moreover, adults are small (Table 3) and pale yellow. It seems that the larvae swallow much of the flour, but they do not digest it completely, perhaps because their principal requirements are proteins and vitamins. To what extent slight fungous growth on dead larvae or on the flour itself is a source of vitamins or energy I do not know.

The addition of blood to wheat flour, however, shortened the larval life of the three Indian rat-fleas a good deal (Tables 1, 2), and a comparatively large number of their larvae completed the active larval life, and without irregularity; this indicates that the mixed diet is in some way more suitable. The fact that a few larvae of *X. cheopis* that bred successfully on blood alone had significantly shorter active larval, and resting larval and pupal life than when its larvae were fed on wheat flour alone, suggests that flea larvae require food rich in nitrogenous substances. The mixed diet of blood and wheat flour, however, lacks some important factors; it is probable that these are vitamins of the B group, and they could not be supplied in adequate amount owing to the desiccating influence of 80 % R.H. on this diet, and also owing to the initial inadequacy of them in the highly milled wheat flour devoid of bran. The comparatively poor development of the larvae of *X. astia* on the mixed diet of blood and wheat flour and on wheat flour alone may indicate that the vitamin B requirements of its larva are exceptionally high.

The adults obtained from the larvae of the three species when nourished on the mixed diet of blood and wheat flour were deep brown and fully sclerotized, unlike those whose larvae fed on wheat flour alone. The absence of proper sclerotization in the adults of *Nosopsyllus fasciatus*, when its larvae were fed on serum and yeast (Sharif, 1937, p. 232), and the development of normal colour in those whose larval diet contained red corpuscles or haemoglobin along with yeast, clearly indicate that these materials contain something, possibly iron, which promotes proper sclerotization. In this connexion it is pointed out that the larvae of *N. fasciatus*, when fed on yeast and serum, to which ferrous ammonium sulphate [$FeSO_4(NH_4)_2SO_4 . 6H_2O$] was added in the same concentration as iron in the haemoglobin, yielded properly sclerotized adults.*

A mixed diet of blood and yeast gave the most rapid development of the larvae of the three species (Tables 1, 2), with almost 100 % emergence of adults, which were normally sclerotized. Possibly owing to the high nutritive value of this mixed diet, it was not

* This fact was not mentioned in my earlier communication (Sharif, 1937), as it escaped my attention then.

necessary for the growing larvae to wait for importation and multiplication of micro-organisms to ensure their successful growth. It is, therefore, evident that this food fulfils the essential requirements of an ideal diet for the larvae of these species. Yeast by itself (Sharif, 1937, p. 233) has no food value for flea larvae. Possibly the yeast proteins are an inadequate diet for them; but yeast possesses growth-promoting substances, the vitamins of the B group. It appears that the native proteins of blood and B vitamins form an ideal food for flea larvae.

There is evidence from insects of many orders of the importance of vitamins of the B group; for the larvae of certain beetles and moths infesting stored products (Fraenkel & Blewett, 1943*a*, pp. 506, 507; Blewett & Fraenkel, 1944, p. 220), and the blow-fly larvae (Hobson, 1933, p. 1908; 1935, p. 1290) cannot grow without them. In these insect larvae the source of these vitamins is micro-organisms which synthesize them in the food. Similarly, it is suggested that when the available food of flea larvae is intrinsically deficient in B vitamins, the association of micro-organisms with it is essential for their normal development.

It seems possible that when fleas feed on a plague-infected rat their excrement, due to the presence of *Pasteurella pestis* (Advisory Committee, 1907*a*, p. 404), may contain materials unusually favourable to their larvae, if they happen to devour that excrement. In my view this may help to explain the great numbers of fleas which have often been observed on rats or trap guinea-pigs in plague-infected houses (see, for instance, Advisory Committee, 1907*b*, p. 443; Hirst, 1926, p. 221). It is, of course, admitted that other factors, e.g. favourable climate and concentration of fleas on a few surviving rats, contribute to the same result (see Hirst, 1926, p. 249; 1927*a*, p. 342); but the phenomenal increase of fleas that is usually found in plague-infected houses cannot be explained by these two factors alone. My assumption is strongly supported by the results of the 'pit experiments' of Webster & Chitre (1930, p. 706) on the transmission of plague by *Xenopsylla astia* and *X. cheopis*. The flea population in the pits increased to such an extent in about a month's time—a period enough for a single generation of fleas in the climate of Bombay—after the introduction of the plague-infected rats, that it caused a heavy mortality amongst rats owing to flea worry and excessive sucking of blood. Heavy infestation of sick rats with fleas, as has been observed by the Advisory Committee (1908, p. 254) and Bacot (1914, p. 472), might be due to the sick rats providing accessory food factors for the larvae of fleas, through the infection of blood with pathogenic micro-organisms.

Flea larvae usually feed on decaying organic matter containing micro-organisms, an association of micro-organisms with the larval food may therefore be essential to some species; but at present the evidence on this point is only indirect. The possibility that flea larvae ingest fungi* has been suggested elsewhere (Sharif, 1948). The exact nutritive value of fungi is as yet unknown; but according to Chapman (1931, p. 159), 'Plant and animal residue may be used more or less directly by the fungi; and the fungi, by the insect.' The fact that premises storing manure, composts and dungs are comparatively more heavily infested with fleas than those without them, indicates that saprophytic fungi may play some part in the increase of fleas.

The existence of 'lagging larvae' of the three Indian rat-fleas on a diet of wheat flour (see p. 255) is definitively suggestive of the occurrence of some gradual and favourable change in this food. Such larvae are the result of malnutrition (Sharif, 1937, p. 232) and not of diapause. The gradual growth of a small quantity of mould on the dead larvae and the food, which perhaps was only sufficient to bring a proportion of larvae to the adult stage, may have allowed others to live much longer than is normal. The marked disparity in growth rate of flea larvae might indicate slow and gradual supply of some other nutritive material to them, in addition to what was present in this food at the beginning of the experiment.

Earlier workers had great difficulty in getting consistent results in breeding flea larvae. In my opinion the partial and variable success in rearing flea larvae obtained by Bacot (1914), Webster (1930) and Sikes (1931) on different diets, but only at a relative humidity of about 70% and higher, was due to the presence of a few micro-organisms, possibly fungi, in the food at some later stage. The fact that a higher percentage of mortality occurred in the beginning of my experiments (see p. 255) supports this assumption.

In nature in a domestic rat burrow I have never come across such an enormous flea population as was found in my breeding jars. Each jar had sea sand and wheat grain, to which a little dried horse blood and yeast was added. For feeding adult fleas a white mouse was put in a jar with a water supply, as described by Leeson (1932, p. 26). The flea population in each jar often increased to such an extent that surplus fleas had to be removed several times a year in order to avoid the death of the mouse. The jar contained only flea and mouse faeces, partially eaten wheat grain, and a small quantity of blood and yeast. In addition, slight putrefaction and fungus growth

* Major W. L. Jellison, of the United States Public Health Service, on a visit to the Haffkine Institute about three years ago, informed me that he has seen on several occasions spores and hyphae of fungi in the alimentary canal of flea larvae. According to him, they are more often found in the gut of the larvae of wild rodent fleas.

was observed in the breeding jars owing to dampness caused by high humidity during the rainy months. The presence of fungus always resulted in excessive increase of the flea population.

A burrow of the Indian domestic rat contains rat and flea faeces, also the nesting material composed of pieces of cloth, gunny bag and paper, strings of cotton, jute and coir, raw cotton, dry leaves and straws of fodder, etc. These items have not much nutritive value for flea larvae; and this probably accounts for a low flea infestation often found in such burrows, especially in dry situations. An appreciable increase in the flea population was observed during the rainy months, when fungous growth on debris and rat faeces in the burrow will occur. Possibly fungous growth, which only occurs at relative humidities higher than 70%, serves an additional source of food. The fact that the larvae of the three Indian rat-fleas (Sharif, 1948) were reared on the mixed diet of blood and yeast at low humidities (50 and 60%), suggests that in the presence of sufficient supply of vitamins of the B group, the association of micro-organisms with the diet to supply accessory food substances is not necessary. It is, therefore, suggested that under dry conditions the source of B vitamins may be different agricultural products that are either found in undigested form in the faeces of their hosts or intact in their burrows. If a larval food with a low concentration of these vitamins is available, as it would be in some rat burrows during dry and hot months, breeding of fleas on a small scale might continue.

VI. INFLUENCE OF LARVAL FOOD ON THE SPECIFIC DISTRIBUTION AND HOST PREFERENCES OF THE THREE INDIAN RAT-FLEAS

The facts of distribution of *X. cheopis*, *X. brasiliensis* and *X. astia* in India are fairly fully known. They may be partly explained by zoogeography. There is no doubt of the fundamental importance of this factor; but it is difficult to see that it could continue to be important for the ectoparasites of the domestic rat which is dispersed so much through human agency. Trade through ships and other modes of transport plays a great part in the dispersal of these rat-fleas, either by transferring them from one place to another or through the agency of their hosts. Climatic conditions prevailing at a place of importation on the bionomics of fleas also control their distribution. I do not find that these factors suffice to explain the presence or absence of the three species of rat-fleas inside India. As fleas are temporary ectoparasites and inhabitants of the nests or retreats of their hosts, many species have become highly specialized not merely in regard to the blood of some particular host, but as to its nesting conditions. It may be that conditions in the burrow make great differences to larval nutrition and therefore to the absence, presence or abundance of flea species. Indubitably, the range of the possible diet of a flea larva is a wide one; but the larvae of all the species are not alike in their food requirements as 'some do not succeed on food that gives good results for others' (Bacot, 1914, p. 513). It is also known 'that no insect can develop on a food which is lacking even in one important constituent' (Fraenkel & Blewett, 1943*b*, p. 485). My own observations in the laboratory on the dietetic requirements of the larvae of different species of the Indian rat-fleas support these assumptions. In the field I have made observations on the rodent biology, which also seem to be relevant.

The examination of about 160,000 fleas of both wild and domestic rodents of the Barsi and Dharwar taluks for about 3 years, and the study of their burrow conditions, have enabled me to determine some factors that control the irregular distribution of the three Indian rat-fleas. These two well-separated taluks lying in the Deccan Plateau have different climatic conditions, Barsi being warmer than Dharwar. *X. cheopis* was predominantly found on the domestic rat, *Rattus rattus rufescens* (Gray), of the Barsi taluk. *Xenopsylla brasiliensis* was present only on the domestic rats, *Rattus rattus rufescens* and *R. rattus wroughtoni* (Hinton), of the cooler uplands of the Dharwar taluk along with *Xenopsylla cheopis*, where infestation of the former flea at some places amounted to 40–56% of the total fleas. But *X. brasiliensis* was almost absent on these domestic rats of some of the adjoining warmer villages, even when situated within about 1–4 miles of those that had this species; in these villages *X. cheopis* was almost exclusively found as in the comparatively warmer regions of this taluk. On the other hand, *X. astia* was present to the exclusion of other fleas on the Indian gerbille, *Tatera indica* (Hardwicke), found in fields, and predominantly on *Bandicota malabarica* (Shaw) in houses, all over these two taluks. *Xenopsylla astia* was also present in very small proportions on the domestic rats of those houses that harboured *Bandicota malabarica*; but it was not found in houses free from this rodent. The fact that only *Xenopsylla cheopis* is found on domestic rats and *X. astia* on *Bandicota malabarica* in the same house, and that the latter flea is the only species found on the wild rodent, *Tatera indica*, in the same village, militates against the idea that the distribution of these fleas is governed exclusively by climatic factors (Taylor & Chitre, 1923, pp. 625–7), or by zoogeographical principles (Hirst, 1927*a*, pp. 317–30). It seems evident that in nature some other biological factor of a greater potency is operating: it is difficult, if not impossible, to escape the conclusion that this factor is the larval food.

The distribution of the three Indian species of *Xenopsylla* and their host preferences can logically be explained on the basis of differences in the nutritional requirements of their larvae. It seems that the larvae of each of these species have certain minimum nutritional requirements. If the food available in a locality is lacking in any one of the required nutritive constituents for the larvae of a species, that insect will be absent. Fleas being mostly inhabitants of the nests and retreats of their hosts, their larvae are dependent upon the nutriment found in them. Organic substances found in the retreats of different rodents are variable (Sharif, 1948). Even in the domestic rat burrow, in addition to flea faeces, the organic matter found, which consists mostly of the nesting material and rat droppings, is very variable in diverse localities, being dependent on the agricultural products available. Furthermore, its nutritive value both in quality and quantity is greatly influenced by the prevailing weather conditions in an area (see Shelford, 1930, p. 101). If the burrow humidity is high, growth of micro-organisms, especially mould, will set in, which will enhance the nutritional value of the larval food. Important articles of diet for rat-flea larvae in nature are rat and flea faeces, but even the nutritive value of the faeces of domestic rats varies in different places. Undigested food particles are passed along with rat faeces; at different places various kinds of crushed grains and their husks, depending on the staple food of the inhabitants of the locality, have been seen in the faeces and stomach contents of rats.

The burrows of *Tatera indica* are very long and tortuous; they are also very deep, their depth varying from 24 to 54 in. The water content of the soil in such burrows must surely be high enough to encourage fungous growth on debris; indeed, I have personally seen moulds growing on the faecal pellets and nesting material of this rodent. It is suggested that the presence of fungus on the debris and litter of such burrows plays an important part in the enormous increase of the population of *Xenopsylla astia* in them. *Tatera indica* usually lives in colonies composed of 6–30 individuals in a complicated burrow system. There can be nothing better for enormous multiplication of *Xenopsylla astia*, as every necessary food constituent for flea larvae is present. Consequently, this flea is abundant in the burrows of *Tatera indica* to the entire exclusion of others throughout the year, and on a number of occasions one to two thousand fleas were recovered from its burrows.

Similarly, many individuals of *Bandicota malabarica* live in a complicated and tortuous burrow system, which is also very deep. The debris in it, especially in the resting chambers, is fairly wet. As a rule, this rodent does not build a nest; only a few sparsely scattered grains, shells of groundnuts, leaves, etc., in a fairly sodden state, with fungous growth on them, were found in the chambers. The flea population, consisting mostly of *Xenopsylla astia*, in each chamber was many times more than was found in a nest of the domestic rat.

Different subspecies of the domestic rat, *Rattus rattus* (Linnaeus), are good and permanent hosts of *Xenopsylla cheopis* in many parts of the world (see, for instance, Advisory Committee, 1908, p. 245; Hirst, 1927*a*, p. 335), especially in the tropical and subtropical countries. Wherever this flea is absent, it is because it is replaced by other hardier species. The depth of the burrows of this rat, examined in the taluks of Barsi and Dharwar, was variable. A typical underground burrow was not very deep, its depth hardly exceeding 20 in. The humidity of such a burrow kept under observation in the town of Dharwar for about a year, fluctuated mostly between 50 and 80%; but it is liable to be lower in dry and higher in damp situations. The chances of fungous growth on the debris and litter of domestic rat burrows in both these taluks appear to be slight. Thus the available food for flea larvae in such burrows is unlikely to be rich in nutriment. Consequently, it might be adequate for the larvae of *X. cheopis*, but not for those of *X. astia*, whose nutritional requirements are higher (see p. 257). A few individuals of *X. astia* were found on the domestic rats of these taluks owing to their close association with *Tatera indica* or *Bandicota malabarica*; but they could not establish themselves on domestic rats on account of the poor larval food available in their burrows.

The association of *Xenopsylla cheopis* with grain in many parts of the world is well known (see Hirst, 1926, p. 163; 1927*a*, pp. 371, 398). The conditions prevailing in most granaries and warehouses are suitable for rearing of this species only (Hirst, 1927*b*, p. 94); in my opinion the debris present here has a low nutritive value and is inadequate for the larvae of *X. astia*. The presence of *X. cheopis* in the stores of polished rice, other grains and cotton in the mercantile premises of Rangoon (Jolly, Fenn & Dorai, 1931, p. 1236), certain trade centres in the plains of the Madras Presidency (King & Pandit, 1931, pp. 366, 369), and Colombo (Hirst, 1927*a*, pp. 344–7, 382; 1933, p. 93), where the indigenous flea on domestic rats found in the residential premises is exclusively *X. astia*, seems to be best explained by the low nutritional requirements of the larvae of *X. cheopis*; as a poor larval diet can only be available in granaries well protected against the humid climatic conditions of these areas. On the other hand, the rat burrows in residential premises in these areas provide a rich source of food on which the larvae of *X. astia* can flourish.

The plateaux of Peninsular India are regions where *X. cheopis* is abundant on domestic rats.

Sorghum (*Andropogon sorghum*), spiked millet (*Pennisetum typhoideum*) and cotton are the main agricultural products of these regions. Cattle fodder is mostly the dried stalks of the two millets, which are stored in stacks in the backyards of houses, and are often utilized by domestic rats to build their nests. The nesting conditions of domestic rats in these regions provide such a poor diet for flea larvae that only *Xenopsylla cheopis* can prosper.

The primary factor affecting the distribution of *X. brasiliensis* is temperature, as the upper temperature limit of its larval development is lower than those of two other species (Sharif, 1948). As mentioned before (see p. 255), its larvae developed best on a diet composed exclusively of wheat flour, which suggests that the vitamin B requirements of the larvae of this species are the lowest. Consequently, this flea mostly abounds in grain stores of cooler places, such as Mysore City, Davanagee, Sagar, Shimoga in the Mysore State (Iyer, 1933, pp. 984–93). This species appears to be common only in the predominantly millet- and rice-growing cooler elevated tablelands of Peninsular India. On the watersheds of the Western Ghats in the Dharwar taluk, it was often found in big commercial towns, but not in the adjoining villages. Its tolerance of a low temperature probably accounts for this disparity in the distribution. Granaries in big towns are well protected against the sun, which is not the case in small villages.

X. brasiliensis is entirely absent in the lowlands of Peninsular India. Its distribution is mainly restricted to the tablelands of Peninsular India and Africa (Buxton, 1941, p. 121), and belts of plains with high temperature have limited its dispersal. Thus its distribution is controlled to a large extent by climatic barriers and zoogeographical principles.

The larvae of *X. astia* are tolerant of a higher temperature than those of the other two species (Sharif, 1948). Patton & Evans (1929, p. 534) suggested that the 'optimum conditions for its life processes are a somewhat higher temperature than *cheopis* and a high atmospheric humidity'. The nutritional requirements of the larvae of *X. astia* being higher than those of the other two species, they require food rich in vitamins of the B group, and possibly in proteins also. Consequently, a higher water content in the food may be needed so as to encourage growth of fungus, which appears to be essential for their development.

X. astia is the flea of domestic rats in rice-growing warm and moist lowlands and plains of India bordering the Bay of Bengal and those of Burma and Ceylon (Hirst, 1927*a*, p. 326). Paddy (unhusked rice) or its husks and straws stored in houses are utilized by domestic rats for making nests. The husk of the paddy is fairly rich in vitamins of the B group, as the pericarp and a greater part of the embryo remain attached to it after the polishing of rice, and in humid and warm places fungus and other micro-organisms can easily flourish on such a litter. Consequently, being a stronger competitor in the struggle for existence than the other two species and able to withstand higher temperature, *X. astia* prospers in it.

Some of the villages of the Dharwar taluk that produced a small quantity of wheat, in addition to other cereals, had comparatively larger proportions of *X. astia* on their domestic rats than those that did not. Possibly, there is some association between wheat cultivation and this flea. McCarrison (1927, p. 636) showed experimentally that of the four important Indian cereals, viz. wheat, spiked millet, sorghum, paddy, the first has more nutritive value both as regards proteins and vitamin B complex, than the others. These cereals can influence the different distribution of rat-fleas by providing larval food of different nutritive value in the burrows of their hosts (see p. 260). In the wheat-growing plains of the Punjab and the United Provinces *X. astia* is also found along with *X. cheopis* on domestic rats in fairly large proportions (Hirst, 1927*a*, pp. 380, 416), and at some places even the former species predominates (Cragg, 1921, 1923). In them wheat chaff and straw are utilized by domestic rats for making their nests, as they are usually stored in houses, and form the main fodder for cattle. Wheat straw and chaff, when kept even in a moderately wet soil, encourage growth of micro-organisms.

In view of this analysis of the burrow conditions of different rodents, it is reasonable to suggest that the irregular distribution of the three species of rat-fleas, especially that of *X. cheopis* and *X. astia*, in India (see Hirst, 1927*a*, p. 381) is governed by the nesting conditions and nature of food of their hosts in combination with the climatic conditions. Wherever debris and litter, containing larval food rich in vitamins of the B group and perhaps in proteins, are present in the burrow of a rodent, either as the result of association of micro-organisms or owing to the presence of substances intrinsically rich in them, *X. astia* is the sole flea. This presumption is supported by the fact that domestic rats in the paddy-growing districts in the warm and moist regions around the Bay of Bengal, and *Tatera indica* and *Bandicota malabarica* in the Deccan Plateau are parasitized by *Xenopsylla astia*. In contrast, if the debris and litter contain larval food with low concentration of these nutriments, as is found in the burrows of the domestic rats of comparatively warmer regions of the Deccan Plateau, *X. cheopis* is exclusively found on them. It is possibly for these reasons that places in the plains of Bundelkhand (Hirst, 1927*a*, pp. 384, 399), the Madras Presidency (Hirst, 1925, p. 10), eastern Bengal, Assam and Burma (Hirst, 1927*a*, p. 392), where the human population lives on locally produced paddy, have *X. astia* as the preponderating species on the domestic rat; but in those places where

the human population subsists on imported polished rice (Hirst, 1933, pp. 93–4), *X. cheopis* is mostly found on this rat. The fact that in Colombo (Hirst, 1927*a*, p. 346), where the domestic rats in the residential areas are exclusively parasitized by *X. astia*, *X. cheopis* is found on rats in the mercantile premises where piece goods, hardware and dry goods are stored, suggests that wherever a poor larval diet is available in the nest of the domestic rat only *X. cheopis* can thrive.

The fact that the larvae of the three Indian species of rat-fleas can develop on proteins other than those of blood (see p. 255) along with vitamins of the B group is of great significance, as it ensures their fairly wide dispersal. The fact that larvae of *X. cheopis* and *X. brasiliensis* develop on wheat flour alone better than those of *X. astia* (Tables 1, 2) affords the first two species better opportunities of establishing themselves when transported with grain far from their original home. It is possibly for this reason that these species have an almost world-wide distribution (Buxton, 1941, pp. 119, 121), and are found in places wherever the environmental conditions are favourable for their breeding. *X. cheopis*, whose original home appears to be the Mediterranean subregion, has extended to moderately cold regions almost all over the world, and has been recorded from places between 40° N. and 40° S. (Sharif, 1930, p. 47). *X. brasiliensis*, which is the second most widely distributed species of the genus (Hirst, 1927*a*, p. 283), having its ancestral home in the plateaux of the Ethiopian region and perhaps of the Ceylonese subregion, has spread to the tablelands of Peninsular India and the seaboard of the continent of America. On the other hand, a comparatively poor growth of the larvae of *X. astia* on wheat flour even in the presence of a slight fungous growth which is only possible at relative humidities higher than 70% (Fraenkel & Blewett, 1943*b*, p. 467), militates against a very wide dispersal of this species through the grain trade in the absence of its hosts. Consequently, *X. astia* has a restricted distribution, confined to the lowlands of the southern Asiatic countries (Hirst, 1926, p. 162), and has not been able to establish itself on rodents of countries far from its original home, even though their climatic conditions may be favourable. Its ancestral home is the Indo-Chinese subregion, and perhaps the Indian and Ceylonese subregions, as it is preponderantly found on their wild rodents.

VII. SUMMARY

The comparative nutritive value of dried horse blood, highly milled wheat flour devoid of bran, a mixed diet of blood and wheat flour and that of blood and yeast for the larvae of *Xenopsylla cheopis*, *X. brasiliensis* and *X. astia* was ascertained experimentally. Pure blood proved inadequate, probably because it is deficient in accessory food factors. The growth of their larvae on wheat flour alone was erratic; only partial success was obtained, and the adults emerged after long and irregular intervals. This I attribute to the association of micro-organisms, possibly fungi, with this food. A mixture of blood and wheat flour quickened their larval development, but it was not a satisfactory larval diet. Blood and yeast form an ideal food for all flea larvae.

It is concluded that larval diets, containing blood or wheat proteins and vitamins of the B group, are essential for the successful rearing of these rat-fleas, and that the proper sclerotization of the adult is due to the presence of haemoglobin in the larval food. The available data on the effects of diverse diets on the growth of flea larvae lend strong support to the conclusion that successful development depends on the presence of these vitamins in the food; it also leads me to think that their source in nature may be the association of micro-organisms with the food.

The larval food appears to be an important factor that governs the distribution and host preferences of different species of flea. The larvae of *X. astia* require the most nutritive diet. If a rich larval food is present in a rodent burrow, *X. astia* flourishes, as in the burrows of *Tatera indica* and *Bandicota malabarica*, and even in those of the domestic rats in certain regions. In contrast, the nutritional requirements of the larvae of *Xenopsylla cheopis* and *X. brasiliensis* are simple; thus they prosper readily in a burrow of the domestic rat, even where the nutritive value of the larval food is very low. As the temperature tolerance of *X. brasiliensis* is the lowest, this species is confined to some of the cooler regions.

The irregular distribution of the three species of rat-fleas inside India may be related to differences in the nutritional value of the varied organic substances found in rat burrows in different places. The fact that the distribution of *X. cheopis* and *X. brasiliensis* is wider than that of *X. astia* is attributed to the ability of the larvae of the first two species to grow better on flour alone; this possibly enables them to survive transport in grain, even without rats, to places far from their original home.

I am much indebted to my chief, Lt.-Col. Sir Sahib Singh Sokhey, Director, Haffkine Institute, Bombay, for affording me many facilities for the pursuit of these investigations, and for his encouragement and advice. Prof. P. A. Buxton, F.R.S., has kindly read through and revised my manuscript and made many valuable suggestions, for which I am obliged to him. I am also obliged to Sir John Taylor for going through the manuscript.

I take this opportunity of acknowledging my

Table 1. *Showing the effects of different foods, when mixed with acid-washed sand, on the growth of the of the three Indian species of* Xenopsylla *at a temperature of* 25 ± 1° C. *and a relative humid*

Species	Larval food used	Experiments tried	No. of larvae used	Larvae died in different active instars: No.	Sig. χ^2 test	Larvae spun cocoons or formed: No. of cocoons	No. of naked pupae	Total	
Cheopis	B.	3	150	145	↑	5	0	5	↑
Brasiliensis	B.	3	165	160	– ↑	0	5	5	– ↑
Astia	B.	4	178	178	+ – ↑	0	0	0	+ – ↑
Cheopis	W.	4	200	125	× × × ↑	75	0	75	× × ×
Brasiliensis	W.	4	196	91	× × × × ↑	105	0	105	× × ×
Astia	W.	4	200	148	× × × + × ↑	52	0	52	× × ×
Cheopis	B. and W.	3	160	0	× × × × × × ↑	1	86	87	× × ×
Brasiliensis	B. and W.	3	155	0	× × × × × × – ↑	0	75	75	× × ×
Astia	B. and W.	4	182	76	× × × × – × × × ↑	3	71	74	× × ×
Cheopis	B. and Y.	3	170	0	× × × × × × × – – × ↑	131	37	168	× × ×
Brasiliensis	B. and Y.	4	204	2	× × × × × × × – – × – ↑	187	15	202	× × ×
Astia	B. and Y.	2	85	0	× × × × × × × – – × – –	81	4	85	× × ×

Table 2. *Showing the effects of different foods, when mixed with acid-washed sand, on the grow Indian species of* Xenopsylla *at a temperature of* 25 ± 1° C. *and a relati*

Species	Larval food used	No. of larvae and period in which they completed their active larval life: Larvae	Days: Range	Days: Mean	Sig. t test	No. of resting larvae and period in which they reached the adult stage: Resting larvae	Days: Range	Days: Mean	Sig. t test	No. of fem of their and: ♀♀	R
Cheopis	B.	5	23–33	29·00	↑	5	12–18	13·80	↑	2	
Cheopis	W.	75	25–59	38·89	× ↑	54	14–26	19·61	× ↑	38	
Brasiliensis	W.	105	21–49	32·46	– × ↑	84	13–24	17·13	× × ↑	49	
Astia	W.	52	44–80	64·62	× × × ↑	41	11–32	16·73	– × – ↑	30	
Cheopis	B. and W.	160	13–21	15·22	× × × × ↑	34	13–17	15·00	– × × + ↑	27	
Brasiliensis	B. and W.	155	13–31	17·74	× × × × × ↑	15	15–19	17·60	× + – – × ↑	9	
Astia	B. and W.	106	20–40	30·63	– × + × × × ↑	44	10–17	13·91	– × × × + × ↑	29	
Cheopis	B. and Y.	170	8–13	10·92	× × × × × × × ↑	164	11–17	13·28	– × × × × × – ↑	83	
Brasiliensis	B. and Y.	202	10–14	11·95	× × × × × × × × ↑	197	11–18	14·35	– × × × – × – × ↑	107	
Astia	B. and Y.	85	10–14	12·24	× × × × × × × × –	85	11–17	13·40	– × × × × × – – ×	51	

Table 3. *Showing the measurements of adults of the three Indian species of* Xenopsylla *reared from with acid-washed sand, at a temperature of* 25 ± 1° C. *and a relative humidit*

Species	Larval food used	No. of ♀♀	Length of females in mm.: Range	Length of females in mm.: Mean	Sig. t test	Breadth of females in mm.: Range	Breadth of females in mm.: Mean	Sig. t test	No. of ♂♂	Le: Ra
Cheopis	B.	2	1·31–1·67	1·490	↑	0·58–0·62	0·600	↑	3	1·35
Cheopis	W.	30	1·04–1·64	1·386	– ↑	0·56–0·84	0·699	– ↑	12	1·04
Cheopis	B. and W.	27	1·27–1·91	1·575	– × ↑	0·67–0·89	0·788	× × ↑	7	1·44
Cheopis	B. and Y.	61	1·76–2·24	1·952	× × × ↑	0·84–1·04	0·950	× × × ↑	61	1·80
Brasiliensis	W.	31	1·09–1·49	1·320	+ + × × ↑	0·58–0·71	0·643	– × × × ↑	30	1·05
Brasiliensis	B. and W.	9	1·25–1·42	1·346	– – × × – ↑	0·53–0·67	0·614	– × × × + ↑	6	1·09
Brasiliensis	B. and Y.	87	1·48–1·88	1·688	× × × × × × ↑	0·72–1·00	0·858	× × × × × × × ↑	67	1·52
Astia	W.	30	1·24–1·49	1·355	+ – × × – – × ↑	0·58–0·75	0·664	+ + × × + × × ↑	11	1·24
Astia	B. and W.	17	1·27–1·91	1·554	– × – × × × × × ↑	0·67–0·89	0·794	× × – × × × × × ↑	15	1·20
Astia	B. and Y.	51	1·76–2·16	1·962	× × × – × × × × ×	0·92–1·16	0·993	× × × × × × × × ×	34	1·84

Key to abbreviations: B., blood; B. and W., blood and wheat flour; B. and Y., blood Sig. t test, t test of significance; Sig. χ^2 test, χ^2 test of significance; –, not significant; ×, significant at 1 % level.

washed sand, on the growth of the recently hatched larvae of 25 ± 1° C. and a relative humidity of 80 %

Larvae spun cocoons or formed naked pupae			Larvae reached the adult stage	
No. of naked pupae	Total	Sig. χ^2 test	No.	Sig. χ^2 test
0	5	↑	5	↑
5	5	− ↑	0	− ↑
0	0	+ − ↑	0	+ − ↑
0	75	× × × ↑	54	× × × ↑
0	105	× × × × ↑	84	× × × × ↑
0	52	× × × + × ↑	41	× × × − × ↑
86	87	× × × × − × ↑	34	× × × − × − ↑
75	75	× × × − − × − ↑	15	+ × × × × × × ↑
71	74	× × × − + × + − ↑	44	× × × − × − − × ↑
37	168	× × × × × × × × × ↑	164	× × × × × × × × × ↑
15	202	× × × × × × × × × − ↑	197	× × × × × × × × × − ↑
4	85	× × × × × × × × × − −	85	× × × × × × × × × − −

with acid-washed sand, on the growth of the recently hatched larvae of the three perature of 25 ± 1° C. and a relative humidity of 80 %

Sig. t test	No. of females	No. of females and duration of their combined larval and pupal life: Days Range	Mean	Sig. t test	No. of males	No. of males and duration of their combined larval and pupal life: Days Range	Mean	Sig. t test
	♀♀ 2	44–45	44·50	↑	♂♂ 3	40–44	41·67	↑
	38	41–75	54·18	− ↑	16	45–65	58·06	× ↑
↑	49	34–62	46·00	− × ↑	35	39–65	49·57	+ × ↑
− ↑	30	60–112	80·17	× × × ↑	11	68–85	76·45	× × × ↑
× + ↑	27	26–33	28·89	× × × × ↑	7	26–33	32·00	× × × × ↑
− − × ↑	9	31–38	33·00	× × × × × ↑	6	28–40	32·50	+ × × × − ↑
× × + × ↑	29	33–48	40·48	− × × × × × ↑	15	37–54	42·40	− × × × × × ↑
× × × × − ↑	83	19–23	21·28	× × × × × × × ↑	81	25–29	27·11	× × × × × × × ↑
× × − × − × ↑	107	21–26	23·08	× × × × × × × × ↑	90	28–32	29·87	× × × × × × × × ↑
× × × × − − ×	51	21–26	23·08	× × × × × × × × −	34	28–31	29·47	× × × × × × × × −

species of Xenopsylla *reared from larvae fed on different foods, when mixed f 25 ± 1° C. and a relative humidity of 80 %*

Sig. t test	No. of ♂♂	Length of males in mm. Range	Mean	Sig. t test	Breadth of males in mm. Range	Mean	Sig. t test
	3	1·35–1·67	1·490	↑	0·58–0·62	0·593	↑
	12	1·04–1·55	1·348	− ↑	0·56–0·69	0·628	− ↑
↑	7	1·44–1·84	1·630	− × ↑	0·58–0·87	0·717	+ + ↑
× ↑	61	1·80–2·12	1·964	× × × ↑	0·72–1·04	0·877	× × × ↑
× × ↑	30	1·05–1·73	1·325	+ − × × ↑	0·53–0·75	0·587	− + × × ↑
× × + ↑	6	1·09–1·42	1·295	− − × × − ↑	0·58–0·69	0·637	− − − × + ↑
× × × × ↑	67	1·52–1·88	1·678	× × − × × × ↑	0·76–0·96	0·814	× × × × × × ↑
× × + × × ↑	11	1·24–1·55	1·400	− − × × − − × ↑	0·53–0·76	0·615	− − + × − − × ↑
− × × × × × ↑	15	1·20–1·91	1·549	− × − × × × × + ↑	0·62–0·87	0·720	× × − × × + × × ↑
× × × × × × ×	34	1·84–2·12	1·968	× × × − × × × × ×	0·76–0·92	0·859	× × × − × × × × ×

l and wheat flour; B. and Y., blood and yeast; W., wheat flour;
of significance; −, not significant; +, significant at 5 % level;

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THE GAMETOCYTOCIDAL ACTION OF PALUDRINE UPON INFECTIONS OF *PLASMODIUM FALCIPARUM*

By P. G. SHUTE and M. MARYON

From the Ministry of Health's Malaria Laboratory, Horton Hospital, Epsom

THE ACTION OF QUININE UPON M.T. GAMETOCYTES

That quinine has little or no destructive action against gametocytes of *Plasmodium falciparum* (crescents) has been known for a long time. Marchiafava & Bignami (1900) pointed out that quinine (grains xxx daily) over a period of time did not perceptibly affect the sexual parasites of *P. falciparum*. Darling (1914), working in the Panama Zone, showed that *Anopheles albimanus* readily became infected when fed upon carriers of M.T. crescents who were taking xxx grains of quinine daily. These and other experiments showed that quinine, even in large doses, has no direct destructive action on crescents, and does not prevent mosquitoes from becoming infected once the crescents are formed and are present in the peripheral circulation. However, quinine given in heavy doses during the first day or two of a primary attack, restricts and reduces the asexual source of supply.

Some workers believe that very small doses of quinine tend to increase the production of gametocytes, and we consider that this is probably correct because patients who receive inadequate treatment during an attack of fever, whereby the asexual forms of the parasite are reduced beyond the pyrogenic threshold but not completely destroyed, frequently produce very large numbers of gametocytes. During recent years we have had the opportunity of observing this effect with a drug other than quinine. Sulphamethazine, given in 4-hourly doses over a period of 24 hr. (10 g. in all), will abort an attack of primary M.T. fever, although usually the period of remission lasts only a few days. Patients whose attacks are aborted two or three times almost invariably develop enormous numbers of crescents.

THE ACTION OF MEPACRINE UPON M.T. GAMETOCYTES

Patients who receive very small doses of mepacrine on the second and third day of a primary attack more frequently develop larger numbers of gametocytes than those who are given a large dose of the drug. This depends partly on the amount of fever preceding the administration of the drug. If, for example, a single dose of mepacrine (0·6–0·8 g.) is given on the second day of a primary attack, crescents are seldom found in large numbers until a fortnight later, as the drug has cut off the source of supply. If mepacrine is given on the sixth or seventh day of fever, even in large doses (0·6–0·8 g.), crescents may be extremely numerous a week or so later. The reason probably is that crescent formation had already started some days previous to the drug being given, and as mepacrine, like quinine, does not sterilize crescents, any already formed, or which were in the process of being formed prior to treatment, would not be affected.

It is interesting to note that as far as can be observed mepacrine does not affect the mature crescents in the peripheral blood. If mepacrine is given when both ring forms and crescents are present, about 10–14 days later it will be seen that while a large proportion of crescents are normal, others are devoid of pigment or may have only one or two specks scattered over the parasite. That these pigment-free sexual forms are virile and infective to mosquitoes has been proved by Sinton (1938). It is believed that the changes occur when the drug is given during the period when crescents are being formed. If mepacrine is given very early in an attack all the ring forms are destroyed before any crescents are formed, while if the drug is given after crescents are formed they are not affected. It is probably only when mepacrine is given during the formation of crescents that these pigment-free parasites are produced, and although it is likely that many of these parasites die, some survive and are infective to mosquitoes. When this occurs, oöcysts are without pigment or contain only one or two specks.

THE ACTION OF PLASMOQUINE UPON M.T. GAMETOCYTES

Barber, Komp & Newman (1929) were among the first to show that plasmoquine, even in very small doses, sterilized the sexual forms of *P. falciparum*. The authors studied stained thin films for evidence of degeneration, faintness of staining, irregularity in the outlines of crescents, vacuolation of the cytoplasm, granulation of the chromatin or displacement of the pigment. Changes, apparently degenerative, appeared in the crescents after plasmoquine treat-

ment, but these could be matched by similar changes in the crescents taken before treatment. They detected one measurable difference: in the films made after plasmoquine treatment a slightly larger percentage of crescents exhibited fainter staining than in the control. They could find no evidence that one sex had been affected by plasmoquine more than the other. Crescents in the shed blood assumed rounded forms after plasmoquine dosage as well as before it, and at least some were alive at a time when they failed to infect mosquitoes. Males exflagellated 24 hr. after plasmoquine treatment, although mosquitoes fed at the time were not infected. The authors concluded that the only reliable test whether crescents were sterilized by drugs was the 'mosquito test', as it was the only one of sufficient delicacy. There was too great a variation amongst normal parasites when stained for this method to prove useful as a test. With this point we are in complete agreement.

THE ACTION OF PALUDRINE UPON M.T. GAMETOCYTES

Hamilton Fairley (1946) and his colleagues, working at Cairns, Australia, have shown that minute quantities of paludrine prevent oöcysts from maturing in mosquitoes even though they have established themselves on the outer wall of the mid-gut of the mosquito.

The following experiment confirms this finding and shows that 0·8 g. of paludrine will prevent mosquitoes from becoming infective (as assessed by the presence of sporozoites in salivary glands) when fed on a patient up to 7 days after the drug had been given. The strain of *P. falciparum* used was the Rumanian, and the mosquitoes were *Anopheles maculipennis* var. *atroparvus*.

The patient was injected intramuscularly with 4 c.c. of infected blood. An incubation period of 4 days followed the injection.

The attack was aborted with paludrine after 5 days of quotidian fever with temperatures between 103 and 104° F. A relapse occurred 3½ weeks later, and on the fourth day quinine was given. Nine days later a further relapse occurred which was again treated with quinine. The third relapse developed 12 days later, and on the eleventh day following this relapse gametocytes first appeared in the peripheral blood. Two days later one dose of 0·4 g. of paludrine was given orally at 1 p.m., and a second dose 2 hr. later. The first batch of mosquitoes was fed 24 hr. later and further batches at 24-hour intervals, with one exception, so long as gametocytes in sufficient numbers (1 in 500 leucocytes) were present in blood films.

Table 1. *Control experiment*

In fresh films exflagellation occurred within 15 min. at 62° F.
140 mosquitoes fed at 11 a.m. Temperature 76° F., humidity 80 %.
No. of gametocytes, 540 males and 1200 females per cu.mm.
Three of the mosquitoes were examined for oökinetes 24 hr. later, and all were positive.

Days after feeding	No. of dissections	Results of dissections
3	3	(2) Numerous oöcysts
		(1) Three oöcysts
4	2	(2) Numerous oöcysts
5	2	(2) Numerous oöcysts
6	2	(1) Few oöcysts
		(1) One oöcyst
7	2	(2) Numerous oöcysts
8	9	(7) Numerous oöcysts
		(2) Negative
9	6	(4) Numerous full-grown oöcysts
		(2) Negative
10	6	(6) Numerous and rupturing oöcysts. Some with sporozoites in the salivary glands

No. dissected 32; percentage positive 87·5

Experiments with paludrine

Two doses, each of 0·4 g. of paludrine, were given orally, one at 1 p.m. and the second at 3 p.m.
In the following experiments exflagellation occurred within 15 min. in fresh films at 62° F., and the conditions of temperature and humidity were similar to those in the control experiment. In each experiment thirty mosquitoes were fed at 11 a.m.

Table 2. *24 hr. after administration of paludrine*

No. of gametocytes, 420 males and 600 females per cu.mm.
Three of the mosquitoes were dissected 24 hr. later and examined for oökinetes. All were positive.

Days after feeding	No. of dissections	Results of dissections
3	6	(1) Numerous oöcysts
		(5) Negative
4	1	(1) Negative
6	4	(2) Many oöcysts
		(1) Few oöcysts
		(1) Negative
		The oöcysts were no larger than is normal for 3 days of growth
8	2	(2) Numerous oöcysts. Some a little larger than normal three-day oöcysts suggesting some slight growth. No disintegration
14	3	(1) Single tiny oöcyst
		(2) Negative
15	3	(1) Over 100 oöcysts (tiny)
		(1) Ten oöcysts (tiny)
		(1) Negative
21	2	(2) Negative

No. dissected 21; percentage positive 42·85

Table 3. *48 hr. after administration of paludrine*

No. of gametocytes, 540 males and 1080 females per cu.mm.
Two of the mosquitoes were dissected 24 hr. later and examined for oökinetes. Both were positive.

Days after feeding	No. of dissections	Results of dissections
3	4	(1) Two oöcysts
		(1) Single tiny oöcyst
		(2) Negative
8	2	(2) Many tiny oöcysts
19	3	(1) Few tiny oöcysts
		(2) Negative
41	3	(3) Negative
42	4	(1) Many tiny oöcysts
		(3) Negative
47	4	(1) Twenty oöcysts
		(3) Negative
		No oöcysts larger than normal 3-day-old oöcysts. Some degenerating, but others appeared normal. Pigment abnormal, misshapen, and had lost the usual pattern

No. dissected 20; percentage positive 35

Table 4. *72 hr. after administration of paludrine*

No. of gametocytes, 360 males and 840 females per cu.mm.
Two of the mosquitoes were dissected 24 hr. later and examined for oökinetes. Both were positive.

Days after feeding	No. of dissections	Results of dissections
3	6	(1) Ten oöcysts
		(5) Negative
8	4	(1) Single tiny oöcyst
		(3) Negative
18	3	(1) Ten oöcysts
		(2) Negative
		Some oöcysts were degenerating

No. dissected 13; percentage positive 23·07

Table 5. 96 *hr.* [4 *days*] *after administration of paludrine*

No. of gametocytes, 540 males and 1320 females per cu.mm.
Two of the mosquitoes were dissected 24 hr. later and examined for oökinetes. Both were positive.

Days after feeding	No. of dissections	Results of dissections
3	3	(1) Numerous oöcysts*
		(1) Four oöcysts
		(1) Negative
		*Pigment scattered over the cysts, capsules crenated, suggestive of disintegration
8	4	(1) Ten oöcysts
		(1) Single oöcyst
		(2) Negative
19	4	(2) Many tiny oöcysts
		(2) Negative
32	4	(3) Less than twelve oöcysts
		(1) Negative

No. dissected 15; percentage positive 60

Table 6. 120 *hr.* [5 *days*] *after administration of paludrine*

No. of gametocytes, 480 males and 600 females per cu.mm.
Two of the mosquitoes were dissected 24 hr. later and examined for oökinetes. Both were positive.

Days after feeding	No. of dissections	Results of dissections
3	3	(2) Many oöcysts
		(1) Negative
17	4	(2) Many tiny oöcysts
		(2) Negative

No. dissected 7; percentage positive 57·1

Table 7. 144 *hr.* [6 *days*] *after administration of paludrine*

No. of gametocytes, 240 males and 300 females per cu.mm.
Two of the mosquitoes were dissected 24 hr. later and examined for oökinetes. Both were positive.

Days after feeding	No. of dissections	Results of dissections
3	4	(2) Ten oöcysts
		(2) Negative
17	3	(1) Numerous oöcysts in every stage of growth up to sporoblast formation
		(1) Single tiny oöcyst
		(1) Negative
36	3	(1) Twenty oöcysts. One oöcyst ¾ grown, capsule crenated and shrunken, degenerating. The rest of the oöcysts were not larger than normal 3-day oöcysts
		(2) Negative
54	5	(5) Negative
84	1	(1) Two tiny degenerating oöcysts

No. dissected 16; percentage positive 37·5

Table 8. 168 *hr.* [7 *days*] *after administration of paludrine*

No. of gametocytes, 240 males and 1080 females per cu.mm.
Two of the mosquitoes were dissected 24 hr. later and examined for oökinetes. Both were positive.

Days after feeding	No. of dissections	Results of dissections
3	2	(2) Ten tiny oöcysts
9	5	(1) Many very tiny oöcysts
		(1) Numerous oöcysts, some half grown
		(3) Negative
24	6	(2) Twenty oöcysts. Some half grown, degenerating; capsules shrunken and crenated
		(1) Numerous oöcysts, some ¾ grown, normal, with one exception, which was degenerating
		(3) Negative
25	2	(1) Ten oöcysts, five ½–¾ grown but degenerating
		(1) Few nearly fully grown oöcysts which appeared to be normal
26	5	(1) Six ½ grown oöcysts all degenerating
		(1) Two tiny oöcysts similar in size to 3-day-oöcysts
		(3) Negative
29	6	(1) Two ½–¾ grown oöcysts
		(1) Six tiny oöcysts
		(4) Negative

No. dissected 26; percentage positive 50

Table 9. 192 *hr.* [8 *days*] *after administration of paludrine*

No. of gametocytes, 600 males and 600 females per cu.mm.
Two of the mosquitoes were dissected 24 hr. later and examined for oökinetes. Both were positive.

Days after feeding	No. of dissections	Results of dissections
3	4	(1) Few oöcysts
		(3) Negative
11	6	(1) Numerous oöcysts small to ¾ grown, apparently normal
		(1) Two tiny oöcysts
		(4) Negative
13	3	(1) Many oöcysts
		(1) Few oöcysts, one fully developed
		(1) Negative
15	3	(1) Many tiny oöcysts, one nearly fully grown. Glands* positive
		(1) Few dozen tiny oöcysts. Glands negative
		(1) One tiny oöcyst. Glands negative
21	3	(1) Five oöcysts, four very tiny, one ½ grown but degenerating
		(2) Negative

* These glands were injected intravenously into a patient but failed to produce infection either clinically or parasitologically.

No. dissected 19; percentage positive 47·36

Table 10. 288 *hr.* [12 *days*] *after administration of paludrine*

No. of gametocytes, 180 males and 300 females per cu.mm.
Two of the mosquitoes were dissected 24 hr. later and examined for oökinetes. Both were positive.

Days after feeding	No. of dissections	Results of dissections
3	2	(2) Few tiny oöcysts
7	4	(1) Ten ½ grown oöcysts, all degenerating
		(3) Negative
9	3	(1) Numerous oöcysts, tiny to fully developed. The mature oöcysts were packed with sporozoites
		(1) Single ½ grown oöcyst
		(1) Negative
17	12	(3) No oöcysts, but sporozoites present in salivary glands
		(9) Negative

No. dissected 21; percentage positive 38·09

11 April 1946. Only six male and 120 female gametocytes per cu.mm.

DISCUSSION

The result of this experiment seems to suggest that the drug destroyed a large number of gametocytes in the blood of the individual. We base this finding on a comparison of the severity of infections in the control batch of mosquitoes with the much lighter infections of the batches fed after giving paludrine. The number of gametocytes did not fall appreciably for at least 9 days following the dose of paludrine, and the small daily variations were within the normal limits. If, as we shall postulate later, it is the female parasite and not the male which is affected by the drug, then one might have expected that by the fourth or fifth day following the administration of the drug the number of females would have been reduced. This did not occur as will be seen in Table 11.

Table 11

Before paludrine given	Males per cu.mm.	Females per cu.mm.
	540	1200
Hr. after paludrine given		
24	420	600
48	540	1080
72	360	840
96	540	1320
120	480	600
144	240	300
168	240	1000
192	600	600
288	180	300
312	50	100
336	10	12

The gametocytes were counted against the leucocytes in thick-thin films which were subsequently stained with either Leishman or Giemsa stain, the number of leucocytes per cu.mm. being a known factor.

The viability of the males was tested daily at the time of feeding the batches of mosquitoes, both in fresh blood and by our routine method of wet preparations as described in an earlier paper (James, 1934). In fresh blood it was observed that most of the males exflagellated readily within 20 min., which is within the normal period. Twenty-four hours after a batch of mosquitoes had fed one or more were dissected and stained smears were made from the blood clot diluted in Locke's solution, and a search made for oökinetes. As long as gametocytes were present in the peripheral blood in sufficient numbers to be detected, and providing they were ripe, oökinetes were formed which appeared to be normal. In the experiment described above no gut dissections for oöcysts were carried out until the third day after feeding. By this time complete digestion of the blood meal had taken place and the oökinetes had penetrated the gut-wall and become established there. They could be seen in the flattened gut with the $\frac{1}{12}$ in. oil immersion lens.

In the control experiment, 87·5 % of the mosquitoes became infected and in many cases the number of oöcysts per gut exceeded 500. The batch which was fed 24 hr. after the administration of paludrine showed a sharp drop in the percentage of mosquitoes infected, but what was of greater significance was the decrease in the number of oöcysts per gut. Oökinetes were plentiful in all the mosquitoes which were dissected 24 hr. after feeding, but despite this, less than half the batch was found to be infected later.

The batch which fed 48 hr. after the administration of paludrine showed a still further drop in the percentage of mosquitoes infected and in no case were there more than twenty oöcysts per gut. The batch which fed on the third day showed a still further decrease in the percentage of mosquitoes infected, and although the numbers of gametocytes remained about the same as before the administration of paludrine, the highest number of oöcysts on any gut was ten as compared with 500 for the controls.

At 96 hr. (4 days) there was a sharp increase in the percentage of mosquitoes which became infected, but there was no appreciable increase in the average number of oöcysts per gut. The batch infected 120 hr. (5 days) after the administration of paludrine showed no change compared with the batch fed on the previous day. It will be seen that among the batch of mosquitoes fed 144 hr. (6 days) after the administration of paludrine, some of the oöcysts, but not all, continued their development in the gut, unlike the oöcysts of the earlier batches. Even in these insects the oöcysts died before complete development took place although they were incubated for 54 days. Very little change was noted in the batch fed 168 hr. (7 days) after the administration of paludrine. The percentage of mosquitoes infected showed a slight increase and some gut infections were heavier as compared with those in mosquitoes fed on the preceding days. None of these oöcysts reached maturity, and in many there was evidence of degeneration as seen by crenation of the capsule and the scattering of the pigment granules over the cysts, a condition seldom if ever seen in normal M.T. oöcysts.

The batch fed 192 hr. (8 days) after the administration of paludrine, was the first to show complete development of sporozoites in the salivary glands. In this batch gland infections occurred in only two mosquitoes out of nineteen, and in these some of the oöcysts failed to grow beyond the very young oöcyst stage, while others which reached a half-grown stage showed signs of degeneration. In this

batch sporozoites were found in the salivary glands on the fifteenth day, only 3 or 4 days later than is normal. The glands and sporozoites of the two mosquitoes were injected intravenously into a patient but the sporozoites were few in number, and the fact that the patient failed to develop fever may have been due to this factor and not to the sporozoites being 'paludrine affected'.

In some of the mosquitoes which fed 288 hr. (12 days) after the administration of paludrine, the oöcysts were fully developed (sporoblast formation), others showed signs of degeneration, and some had not developed beyond the rounding off stage after penetrating the gut wall, even after the ninth day of incubation.

In searching for an explanation of the action of paludrine on gametocytes Hamilton Fairley (1946) and his colleagues suggest that it is not until the gametocytes get into the gut of the mosquitoes that the drug acts on them. If this is the case then it would seem that it may be when the gametocytes are released from the erythrocyte that the drug acts. However, we suggest that it is the females, and not the males, which are acted upon and that the action is a slow one. We base this belief upon the following observations. The males exflagellate normally in freshly drawn blood and also in the blood clot in the gut of the mosquito. The females become fertilized in the blood clot and some, at least, succeed in penetrating to the outer membrane of the gut of the mosquito.

We attach greater importance to the actual numbers of oöcysts per gut than to the percentage of mosquitoes infected. Paludrine appears to act very slowly on the female parasites and it seems that a large number are destroyed or sterilized before they are fertilized or before they are able to penetrate the wall of the gut. This might explain the sharp fall in the number of oöcysts per gut compared with the controls, especially as there was no appreciable decrease in the numbers of gametocytes in the peripheral blood. The females which are not completely sterilized before fertilization are, we believe, drug-affected (sick parasites), and because the action of the drug is slow, some females survive long enough to penetrate the wall of the gut but subsequently die. Some die almost as soon as they reach the gut wall and others a few days later, depending on the number of days which have elapsed since the drug was given to the patient.

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